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GENETIC TOXICOLOGY OF PYRVINIUM PAMOATE

by

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SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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DATE 16 August 1984

Dedicated to my mother,

who never doubted that I could carry out

scientific endeavors,

even under 'combat' conditions

ABSTRACT

The genetic activity of several anthelmintic drugs that are used routinely in oxyuriasis therapy was studied in diploid mitotic recombination and gene conversion assays (strains D5 and D7 of *Saccharomyces cerevisiae*), and in haploid yeast reversion assays (strains XV185-14C, XY718-1A, and 7854-1A). Piperazine citrate, piperazine adipate, mebendazole, thiabendazole, and pyrantel pamoate did not appear to be active genetically in yeast. Pyrvinium chloride, pyrvinium iodide, and all of the medical grades of pyrvinium pamoate were recombinogenic in strains D5 and D7, and mutagenic in strains XV185-14C, XY718-1A, and 7854-1A. The degree of genetic activity varied considerably among the medical grades of pyrvinium pamoate that were tested. Similarly, these medical grades also varied in the degree of mutagenicity when they were tested in strains TA98 and TA100 of *Salmonella typhimurium* but some of the medical grades of pyrvinium pamoate were mutagenic in the presence and in the absence of the metabolic transformation system, whereas other medical grades of the drug required such activation to be mutagenic. Monopotassium pamoate was not mutagenic in yeast. Light-catalyzed degradation did not enhance the genetic activity of pyrvinium pamoate; the degraded samples of pyrvinium pamoate were not mutagenic.

With the use of a thin-layer chromatographic detection technique, samples of pyrvinium pamoate from several sources were found to contain different numbers and quantities of impurities. In addition, the medical grades and dosage forms of several brands of pyrvinium pamoate were analyzed by fluorescence high pressure liquid chromatography to determine the relative quantities of the impurities in these pharmaceuticals.

In general, there is a correlation between the degree of genetic activity and toxicity, and the number and relative quantity of impurities in each medical grade of pyrvinium pamoate.

An investigation of the structural requirements for the mutagenic action of pyrvinium salts was carried out with the use of the 6-chloro- and 6-methyl-analogs of pyrvinium iodide. The genetic activity of these analogs was studied in diploid and haploid yeast in order to gain information about the structural features that are required for the genetic activity of pyrvinium. A methyl- or a dimethylamino-substituent at the 6-position and the cationic site at the methylated ring nitrogen are required for mutagenic activity. The cationic site appears to be essential. Petites were induced in *Saccharomyces cerevisiae* (strains D5 and N123) with the pyrvinium salts and the analogs. The dipyrvinium salt (pyrvinium pamoate) was more efficient than the monopyrvinium salt (pyrvinium iodide) and its analogs at inducing petites. The primary mechanism of action of these anthelmintic drugs may be the destruction of the mitochondrial DNA of helminths.

The genetic activity of pyrvinium pamoate was also evaluated *in vivo*, and the drug induced elevated levels of micronuclei in the bone marrow cells and nuclear damage in the colon cells of mice. Low levels of nuclear aberrations were observed in the colon cells of mice after the administration of oral suspensions of pyrvinium pamoate (Vanquin® and Pyr-Pam®) that are available commercially in Canadian pharmacies.

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INTRODUCTION

A heritable change in cell regulation is a characteristic property of a cancer cell, and cell regulation can be altered by mutation. Mutagenicity assays, which can be performed rapidly only in microbial systems, are used to identify potential mutagens. One of the first, and the most frequently used, of the short-term mutagenicity assays utilized the enteric bacterium *Salmonella typhimurium* (Ames, 1971; Ames *et al.*, 1972). Each strain in the *Salmonella typhimurium* tester series contains a different type of mutation in the histidine operon; the histidine operon has a cluster of 9 genetic loci. *HisG* encodes the enzyme phosphoribosyladenosine triphosphate pyrophosphorylase, which mediates the production of phosphoribosyladenosine triphosphate from adenosine triphosphate and phosphoribosylpyrophosphate; this is the first step in histidine biosynthesis. *HisD* is the structural gene for the enzyme histidinol dehydrogenase, which converts L-histidinol to L-histidine.

The missense mutation *hisG46* in strain TA100 results in the substitution of proline ($\frac{-GGG-}{-CCC-}$) in place of leucine ($\frac{-GAG-}{-CTC-}$), and this has been determined by DNA sequence analysis (Barnes *et al.*, 1982). The *hisD3052* mutation in strain TA98 is a -1 base-pair deletion (Isono and Yourno, 1974). The site of this frameshift mutation is near a series of alternating guanine and cytosine residues with the sequence $\frac{-GCGCGCGC-}{-CGCGCGCG-}$. This strain usually reverts by -2 base-pair deletions, which restore the reading frame. The reversion to his^+ in these strains of *Salmonella typhimurium* can occur at the locus (the site of the original mutation) or at a suppressor (Walker, 1982).

Salmonella typhimurium has a lipopolysaccharide coat that is relatively impermeable, particularly to compounds of high molecular weight, in the wild-type bacterium. The *rfa* mutation, or "deep rough" character,

causes a partial loss of the lipopolysaccharide surface of the bacterial membrane and this facilitates the entry of large molecules that normally do not penetrate the cell wall (Maron and Ames, 1983). The tester strains have a defective lipopolysaccharide core, in which the proximal and distal heptoses are not present, and this is a result of a mutation that involves the *rfaE* and *rfaF* loci (Ames *et al.*, 1973b). The bacterial tester strains also contain a mutation that eliminates the accurate repair of lesions in the DNA, and this enhances the sensitivity of the test. The mutation (*ΔuvrB*) is a deletion of the gene that encodes an endonuclease that is involved in excision repair. This endonuclease recognizes lesions that distort the DNA helix and the absence of the enzyme results in the increased mutagenic repair of these lesions (Walker, 1982; Maron and Ames, 1983). The deletion (*ΔuvrB*) extends through the biotin operon, thus the tester strains have a nutritional requirement for biotin.

Bacterial cells that harbor the pKM101 plasmid are more susceptible to frameshift and base-substitution mutagenesis. The plasmid-containing cells appear to have an increased ability to undertake the error-prone repair of DNA lesions. The pKM101 plasmid contains the *bla* gene, which encodes the enzyme β-lactamase. This enzyme is responsible for the antibiotic resistance to ampicillin and other penicillins with a lactam ring system. This plasmid also contains the *muc* genes, which are believed to encode an endonuclease that is active on single- and double-stranded DNA (Langer *et al.*, 1981). The pKM101 plasmid confers increased susceptibility to UV and chemical mutagenesis, resulting in increased frequencies of transitions and transversions. This plasmid appears to encode proteins that are involved in error-prone repair

(Walker, 1982). Analyses of the mechanism by which the pKM101 plasmid enhances mutagenic repair have been carried out in *Escherichia coli*. The plasmid encodes the *mucA* and *mucB* genes (*muc* is an acronym for mutagenesis: UV and chemical), which are analogs of the chromosomal genes *umuD* and *umuC* (*umu* is an acronym for UV mutable). These chromosomal genes are required for UV and chemical mutagenesis (Walker, 1984). DNA damage induces the expression of both the *mucA/B* and *umuD/C* loci. Strains TA98 and TA100 of *Salmonella typhimurium* carry the pKM101 plasmid, which enhances the capacity for mutagenic repair and, when coupled with the reduction in the function of the excision repair system (*ΔuvrB*), produces tester strains that are sensitive to a wide array of mutagens.

The bacterial tester strains are useful in determining the mechanisms of mutagenesis of chemicals. A difficulty that arose with these assays was that bacteria do not have the enzymes that are involved in the metabolism of drugs in mammals, therefore liver microsomes were added to the bacteria, *in vitro*, to detect mutagenic metabolites (Ames *et al.*, 1973a). The prototype for the metabolic activation of chemical carcinogens is 2-acetylaminofluorene (Figure 1). This aromatic amine is a carcinogen and it induces tumors in all animal species that have been tested except the guinea pig, which cannot convert 2-acetylaminofluorene into the active metabolite (Bates, 1977). 2-Acetylaminofluorene is metabolized to *N*-hydroxy-2-acetylaminofluorene by hepatic enzymes (Cramer *et al.*, 1960) and this metabolite is converted into the ultimate carcinogen, 2-acetylaminofluorene-*N*-sulfate, by sulfate conjugation in the liver (DeBaun *et al.*, 1970a,b; Weisburger *et al.*, 1972). The ultimate carcinogen, 2-acetylaminofluorene-*N*-sulfate, was demonstrated to be

a mutagen in *Bacillus subtilis*, but 2-acetylaminofluorene and its *N*-hydroxylated metabolite were not mutagenic (Maher *et al.*, 1968); this assay did not include the addition of mammalian enzymes. In *Salmonella typhimurium*, 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene were not mutagenic unless liver microsomes were added (Ames *et al.*, 1972; McCann *et al.*, 1975). Electrophilic metabolites such as 2-acetylaminofluorene-*N*-sulfate appear to be the active forms of most chemical carcinogens and many chemical mutagens. These electrophilic reactants combine covalently with nucleophiles in DNA, RNA, and proteins. The cellular target of mutagens is the DNA.

Tissue homogenates from mammals, usually rodents, provide the metabolism in the bacterial assays. This *in vitro* activation complex requires an NADPH-generating system plus an extract of liver microsomes (the S9 fraction). For maximum enzymatic activity, the mammalian liver microsomes must be induced with aromatic hydrocarbons such as Aroclor 1254, which is a mixture of polychlorinated biphenyls. The standardized method for the preparation of tissue homogenates, as described by Maron and Ames (1983), involves the administration of an enzyme inducer (200 mg of Aroclor 1254/ml of corn oil) to male Sprague-Dawley rats by a single, intraperitoneal injection. The rats are killed by cervical dislocation 5 days after the injection, the desired tissue (usually the liver) is homogenized, and the homogenate is centrifuged at 9000 g for 10 minutes. The supernatant (the S9 fraction), which contains the microsomal enzymes, is decanted, frozen immediately, and stored at -80°C.

Most xenobiotics undergo oxygenation (Phase I metabolism) and conjugation (Phase II metabolism) to produce polar metabolites that are sufficiently water-soluble for renal excretion. Polysubstrate mono-

oxygenases are Phase I enzymes that metabolize most carcinogens and mutagens; cytochrome P-450 is a membrane-bound, multicomponent enzyme system that requires molecular oxygen and NADPH.

The yeast *Saccharomyces cerevisiae* is an ideal organism for studying the effects of mutagens and carcinogens since it is able to activate promutagens via its cytochrome P-450 system (Callen and Philpot, 1977; Callen, 1978). Yeast has a true nucleus, 17 chromosomes (Mortimer and Schild, 1981), introns, and mitochondria. As a result of the haploid and diploid cell cycles, yeast can be representative of prokaryotes (bacteria) and higher eukaryotes (animals) in many respects. An additional advantage is that the diploid cell can undergo meiosis.

In order to detect as many different types of mutational lesions as possible, mutant alleles of various genes in several metabolic pathways were assembled in one strain of *Saccharomyces cerevisiae*. Strain XV185-14C (constructed by S-K. Quah and R.C. von Borstel; cited in von Borstel, 1981) has the genotype

a *ade2-1 arg4-17 lys1-1 trp5-48 his1-7 hom3-10*.

ADE2 (on linkage group 15R) encodes phosphoribosylaminoimidazole carboxylase, which is involved in purine metabolism; *ARG4* (on linkage group 8R) encodes L-argininosuccinate lyase, which cleaves L-argininosuccinate to produce L-arginine; *LYS1* (on linkage group 9R) encodes saccharopine dehydrogenase, which mediates the oxidation of saccharopine, with the release of α-ketoglutarate, to yield L-lysine; *TRP5* (on linkage group 7L) encodes tryptophan synthase, which mediates the condensation of L-serine with indole-3-glycerol phosphate, with the release of glyceraldehyde-3-phosphate, to produce L-tryptophan; *HIS1* (on linkage group 5R) encodes ATP-phosphoribosyltransferase, which is involved in the

condensation of the ribosylphosphate moiety of phosphoribosylpyrophosphate onto ATP to yield phosphoribosyl-ATP; *HOM3* (on linkage group 5R) encodes L-aspartate kinase, which mediates the reaction between L-aspartate and ATP to produce β-aspartylphosphate. The *hom3-10* mutant requires L-homoserine or both L-methionie and L-threonine because of the existence of a common pathway, via homoserine, for the biosynthesis of these amino acids.

Strain XV185-14C was constructed specifically to encompass the features of several mutant alleles: *ade2-1*, *arg4-17*, *lys1-1*, and *trp5-48* are suppressible, chain-terminating mutants, *his1-7* is a missense mutant that is not suppressible by external suppressors, and *hom3-10* is believed to be a frameshift mutant.

The chain-terminating mutants are known to be of the ocher (UAA) type. This has been determined by back-translation from amino acid sequences of revertant iso-cytochrome *c* (Gilmore *et al.*, 1971) and by noting the efficient reading, *in vitro*, of an ocher nonsense codon contained in a messenger RNA (Gesteland *et al.*, 1976). The DNA of an ocher nonsense suppressor, *SUP4*-o, was sequenced (Goodman *et al.*, 1977) and the mutant site was found to be at the wobble position of a $tRNA^{Tyr}$. This provided the biochemical evidence that confirmed the genetic argument for the association of yeast nonsense suppressors with mutant tRNAs, which was put forward by Magni *et al.* (1966). The mutant site that was identified by Goodman *et al.* (1977) had a thymine in place of guanine, and this suggests that the base in the wobble position, which was responsible for the ocher-suppressing phenotype, was a modified pyrimidine such as a 2-thio-5-carboxymethyluridine derivative or a 5-carboxymethyluridine (Sherman, 1982). From the considerations that

have been outlined, the types of reveratan of *ade2-1*, *arg4-17*, and *lys1-1* can be deduced: the 5'-UAA-3' ocher nonsense codon could revert to either 5'-UAU-3' or 5'-UAC-3', both of which are tyrosine codons, and the anticodon of the $tRNA^{Tyr}$ would mutate from 5'-GΨA-3' to 5'-ZΨA-3', where Z is the unknown modified uridine and Ψ is pseudouridine. Therefore, reversions at the locus comprise AT→TA and AT→CG transversions (von Borstel *et al.*, 1973), whereas reversions in the anticodon of the suppressor $tRNA^{Tyr}$ are GC→TA transversions.

The missense mutant *his1-7* has a spontaneous reversion rate that is nearly the same as the forward spontaneous mutation rate from canavanine sensitivity to resistance (Gottlieb and von Borstel, 1976). Most of the reversions occur by second-site mutations in the *HIS1* locus (Lax and Fogel, 1978). The reversion of *his1-7* by intragenic missense suppression is also evident from the phenotypic heterogeneity of the revertants, since many of them are feeder colonies. Missense mutants are believed to arise by base-substitution mutations. The *his1-7* mutant is osmotically remediable by 1 M KCl when grown at 18°C, but not at 25°C or 33°C (Hawthorne and Friis, 1964); the *his1-7* mutant has the same spontaneous reversion rate during mitosis and meiosis (S. Fogel and D.D. Hurst, unpublished data); and *his1-7* is highly revertible with ethylmethane sulfonate (Shahin and von Borstel, 1978), which primarily induces transitions in phage T4 (Krieg, 1963) and in *Neurospora crassa* (Malling and de Serres, 1968). In yeast, *his1-7* is also reverted by the mutator *mut7,8*, which appears to induce preferentially AT→TA and AT→CG transversions (D.S. Bendiak and J.M. Tyler, unpublished data). The evidence, when compiled, indicates that *his1-7* is a missense mutant that can revert by transitions or transversions.

The *hom3-10* mutant was postulated to be a frameshift mutant because its spontaneous reversion rate during meiosis is about 30 times higher than during mitosis (Magni, 1969). In addition, *hom3-10* responds to frameshift suppressors (G. Lucchini Bonomini, unpublished data).

Strains XY718-1A (genotype a *his4-519 leu2-3*) and 7854-1A (genotype a *his4-38 leu2-3*) are particularly useful for detecting the specificity of frameshift mutagens. *HIS4* (on linkage group 3L) encodes a trifunctional poly peptide: *HIS4A*, phosphoribosyl-AMP cyclohydrolase; *HIS4B*, phosphoribosyl-ATP pyrophosphohydrolase; and *HIS4C*, histidinol dehydrogenase. The mutations mapped to the *HIS4A* region and are designated as *his4A-519* and *his4A-38* (Culbertson *et al.*, 1977). *LEU2* (on linkage group 3L) encodes β-isopropylmalate dehydrogenase, which converts β-isopropylmalate to α-ketoisocaproate.

The frameshift mutants *his4A-519*, *his4A-38*, and *leu2-3* were induced by mutagenesis with ICR-170, which is an acridine half-mustard (Culbertson *et al.*, 1977). All 3 mutants have +1 base-pair (G·C) additions in regions of DNA with multiple G·C base pairs, and these frameshift mutations can be suppressed by frameshift suppressors that recognize 4-base glycine codons (Donahue *et al.*, 1981, 1982). The concomitant reversion to a His^+Leu^+ phenotype occurs by the induction of external frameshift suppressors (Culbertson *et al.*, 1977, 1980), and altered tRNAs ($tRNA^{Gly}_{CCCC}$ and $tRNA^{Gly}_{CCCA}$) mediate frameshift suppression by reading these 4-base codons (Donahue *et al.*, 1981).

Diploid strains of *Saccharomyces cerevisiae* are used to detect the genetic activity of mutagens via chromosome breakage that leads to mitotic recombination. Strains D5 (genotype $\frac{\alpha}{a}\ \frac{trp1\text{-}1}{+}\ \frac{ade2\text{-}40}{ade2\text{-}119}\ \frac{MAL1}{+}\ \frac{+}{MAL4}$) and D7 (genotype $\frac{\alpha}{a}\ \frac{ade2\text{-}40}{ade2\text{-}119}\ \frac{trp5\text{-}12}{trp5\text{-}27}\ \frac{ilv1\text{-}92}{ilv1\text{-}92}$) are particularly useful

(Zimmermann, 1973; Zimmermann *et al.*, 1975). The designations a and α represent complementary mating-type alleles; *trp1-1* (on linkage group 4R) is an amber (UAG) nonsense mutant that terminates the production of phosphoribosylanthranilate isomerase; *MAL1* (on linkage group 7R) and *MAL4* (on linkage group 11R) are characterized by constitutive maltose fermentation, and the gene products are believed to be involved in the regulation of α-glucosidase synthesis.

ILV1 (on linkage group 5RO encodes theonine deaminase, whidh mediates the deamination of threonine to yield α-ketobutyrate in the first step of isoleucine biosynthesis; the *ilv1-92* mutant is used for detecting the induction of reverse mutations. Strain D7 is heteroallelic for the noncomplementing alleles *trp5-12* and *trp5-27*, and prototrophs can arise by reciprocal intragenic recombination or by nonreciprocal mitotic recombination (referred to as gene conversion), which are indistinguishable phenotypically. For practical purposes, Zimmermann *et al.* (1984) suggest that the generation of tryptophan prototrophs should be referred to as gene conversion.

Strains D5 and D7 are heteroallelic for two complementing mutations of the *ADE2* locus: an allele with a red phenotype and an absolute requirement for adenine, *ade2-40*, and a slightly leaky allele with a pink phenotype, *ade2-119*. The red pigment that accumulates in *ade2* mutants results from the polymerization of phosphoribosylaminoimidazole, which is the substrate for the gene product of the *ADE2* locus. When the *ade2-40* and *ade2-119* alleles are present in the same diploid strain, they complement each other and, as a result, white colonies are formed.

A mitotic crossover event between the centromere and the *ade2* locus results in the formation of a twin-spot, which is a colony with red and

pink sectors. An entirely red or an entirely pink colony can arise when mitotic gene conversion, mutation, or nondisjunction has taken place. Other aberrant colonies, such as those with red-pink-white, red-white, or pink-white phenotypes may be produced when mitotic recombination, mitotic gene conversion, mutation, or nondisjunction has taken place in one of two or more cells together in a cluster at the time of mutagenization.

The information on the gene products that are encoded by the various genetic loci, and the biosynthetic and metabolic pathways, of the yeast *Saccharomyces cerevisiae* has been obtained from the data that was compiled by Plischke *et al.* (1976), and by Jones and Fink (1982).

Just as the *in vitro* activation system that is used with *Salmonella typhimurium* does not contain the particular enzymes or cofactors that are required for all of the possible activations that would take place *in vivo*, yeast cannot carry out the entire spectrum of drug metabolism that would be expected in mammals, *in vivo*. The susceptibility of a tissue to a particular carcinogen or mutagen may be determined by the type of metabolizing enzymes that are present in that tissue. The differences in the activating capabilities of S9 fractions from different tissues, as well as from different species, attest to this tissue specificity (Haroun and Ames, 1981).

Carcinogens that are difficult to identify in microbial systems are those that are unique to animals. For example, the action of hormones or analogs of hormones (i.e., diethylstilbestrol), are unique to animals as they must interact with specific receptors, and this results in more stringent structure-activity relationships than those for an interaction of an electrophilic reactant with DNA.

It is not possible for one assay to detect every mutagen or carcinogen, therefore it is desirable to use a battery of tests that includes bacteria, yeast, mammalian cells in culture, and animals. Generally, the results that are obtained with yeast are indicative of those that might be obtained with mammalian cells in culture. In fact, yeast is preferable because a yeast cell is a single organism, whereas tissue cultures exist in an environment that is removed from the influences of adjacent tissues and organs that are present in live animals. As a result of the artificial environment of mammalian cells in culture, tissue cultures are prone to the generation of artefacts and this must be taken into account when mutagens and carcinogens are being evaluated.

STATEMENT OF THE PROBLEM

It has been established that pyrvinium pamoate (Figure 2) is a mutagen in bacteria. This anthelmintic drug induces frameshift and base-substitution mutations in *Salmonella typhimurium*, but only when the metabolic activation system (the S9 microsomal fraction of mammalian liver) is added to the assay (MacPhee and Podger, 1977). Since pyrvinium pamoate is not absorbed appreciably from the gastrointestinal tract (Rollo, 1980), it cannot reach the liver, where the metabolism of promutagens to mutagens takes place. Thus, it is believed that the mutagenic effects of this drug on mammals are negligible.

The mutagenicity of pyrvinium pamoate in bacteria was considered to be of sufficient importance to examine the drug in a microbial assay that could detect chromosome breakage, as well as frameshift and reverse mutations. The yeast *Saccharomyces cerevisiae*, which is capable of activating promutagens into mutagens via its cytochrome P-450 system (Callen and Philpot, 1977; Callen, 1978), is an ideal organism for studying the genetic activity of chemicals. Pyrvinium pamoate induced frameshift and base-substitution mutations in haploid yeast, confirming the results that were obtained with the bacterial assay, and the drug also induced chromosome breakage in diploid yeast.

The medical grades of pyrvinium pamoate that are marketed in Mexico, and one medical grade that is marketed in Canada, are mutagenic in *Salmonella typhimurium* in the absence of the metabolic transformation system (Cortinas de Nava *et al.*, 1983; Hennig *et al.*, 1984). The enhanced genetic activity of these medical grades was thought to be due to the presence of one or more impurities. This required an evaluation

of the chemical purity of the medical grades of pyrvinium pamoate that are used to formulate the dosage forms of the drug. Also, the identification and quantitation of the mutagenic impurities were of importance. Mutagenic impurities have been reported for other chemicals (McCann and Ames, 1977).

Due to the existence of medical grades of pyrvinium pamoate that do not require metabolic activation to be mutagenic (Cortinas de Nava *et al.*, 1983; Hennig *et al.*, 1984), it was essential to study pyrvinium pamoate more closely for its molecular action, its effect on cell organelles, and its activity in mammalian cells *in vivo*. Chromosome breakage in yeast is an indication that pyrvinium pamoate may also damage the genetic material of mammalian cells. In comparison to the other anthelmintic drugs that are available, pyrvinium pamoate, which is an over-the-counter product, has the fewest side effects. In children, pyrvinium pamoate is the mainstay of oxyuriasis therapy, thus it was imperative to analyze this drug for potentially harmful effects.

Pyrvinium pamoate was not active genetically in mammalian cells in culture (Lake and de la Iglesia, 1981), and mammalian (human and mouse) urine samples that were taken after oral doses of the drug were not mutagenic in *Salmonella typhimurium* (Lake and de la Iglesia, 1981; Cortinas de Nava *et al.*, 1983). Although these results suggested that the drug was not harmful, it was important to evaluate pyrvinium pamoate *in vivo* in mammalian assays that reflect both the metabolism and the tissue specificity that this anthelmintic drug would undergo in humans.

The 'shotgun' treatment of mice, by intraperitoneal injection rather than by oral intubation, with pyrvinium pamoate and other drugs

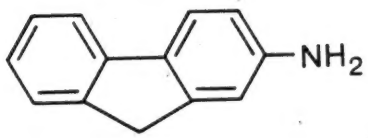
in the bone marrow assay is carried out to gain an immediate response or answer as to whether or not a drug is active genetically in mammals. Aside from being a valid procedure for examining the genetic effect of monopyrvinium salts, which are absorbed systemically, the results of a 'shotgun' treatment can provide enough information to facilitate the design of future animal experiments. Experimentation with animals is usually undertaken only when there is a reasonable expectation that the results will lead to an improvement in the health and welfare of people. The results of experiments using pyrvinium pamoate in live animals appeared to validate the results that were obtained with the bacterial and yeast assays. This is essential since the use of reliable microbial systems minimizes the requirement for the use of large numbers of animals in validation experiments.

Microbial systems, even with the concomitant use of the *in vitro* liver homogenate, cannot be a substitute for the absorption, distribution, metabolism, and excretion that occurs in animals. An important example is the analgesic drug phenacetin, which is metabolized to the ultimate carcinogen/mutagen via conjugation that is mediated by sulfotransferases. Most mammals do not utilize sulfate conjugation as a primary mechanism in Phase II metabolism, however, if high doses of phenacetin are given chronically, sulfotransferases are induced and undertake a large proportion of the conjugation reactions. Hamsters utilize sulfate conjugation as a primary method of metabolism, thus phenacetin is mutagenic in *Salmonella typhimurium* when hamster S9, not rat S9, is added (Weinstein *et al.*, 1981).

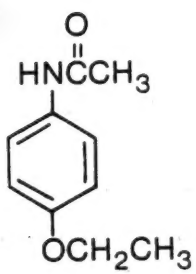
An examination of analogs and the probable metabolites of a potential carcinogen such as pyrvinium pamoate is important because the

mutagenic properties of most chemical mutagens are related to their electrophilic reactivity, which is associated with specific functional groups. The 6-dimethylamino-substituent of pyrvinium pamoate (see Figure 2) is a potential site for the conversion to an electrophilic metabolite.

The mutagenic potencies of chemicals in bacteria and yeast may parallel their carcinogenic potencies in mammals. According to McCann and Ames (1977), 90% (157/175) of the carcinogens that have been tested in *Salmonella typhimurium* were mutagenic, and 87% (94/108) of the non-carcinogens were nonmutagenic. This indicates that the Salmonella/-microsome test has an accuracy of approximately 90% in the detection of carcinogens as mutagens. An accuracy of 90% for this bacterial assay suggests that the results of a combination of bacterial, yeast, and animal studies can be extrapolated to humans to predict the toxicity of a drug such as pyrvinium pamoate with an acceptable degree of certainty.



2-aminofluorene (MW 181.22)



phenacetin (MW 179.21)

FIGURE 1. The prototypes for the metabolic activation of chemical carcinogens in mammals are 2-aminofluorene, its metabolite 2-acetylaminofluorene, and the analgesic drug phenacetin.

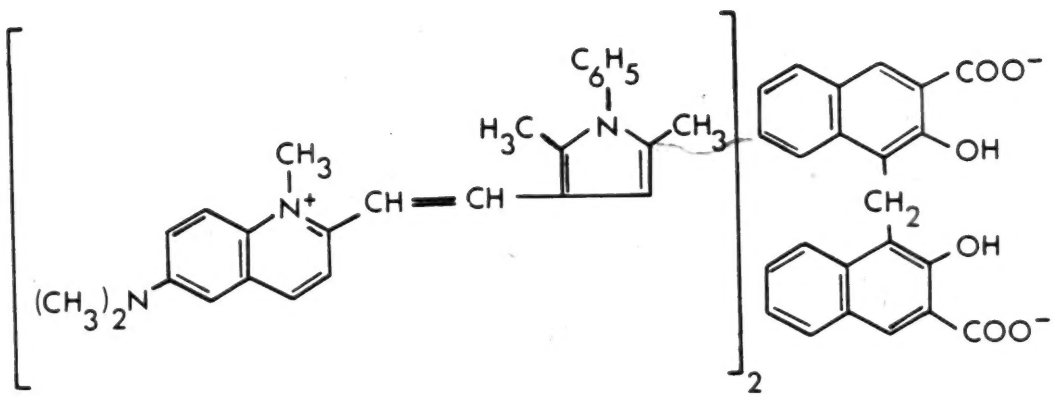


FIGURE 2. The structure of the dipyrvinium salt, pyrvinium pamoate (MW 1151.44). Pyrvinium chloride (MW 417.99) and pyrvinium iodide (MW 509.45) are monopyrvinium salts.

METHODOLOGY

REFERENCE STANDARDS AND DOSAGE FORMS

The USP reference standard of pyrvinium pamoate (lot F2) was purchased from the Drug Standards Division (US Pharmacopeial Convention, Incorporated), the medical grades of pyrvinium pamoate were supplied by Parke-Davis Canada Incorporated, Charles E. Frosst & Company, ICN Canada Limited, and Laboratorios Columbia. Vanquin® (Parke-Davis Canada Incorporated), Pamovin® (Charles E. Frosst & Company), and Pyr-Pam® (ICN Canada Limited) were provided by the manufacturers of these products. The labeled strengths of the dosage forms are 75 mg pyrvinium pamoate/tablet (equivalent to 50 mg of pyrvinium base) and 15 mg pyrvinium pamoate/ml suspension (equivalent to 10 mg of pyrvinium base).

Original samples of pyrvinium chloride (Parke-Davis Canada Incorporated) and pyrvinium iodide (Delmar Chemicals, Montreal) were gifts from H.D. Beckstead (Drug Identification Laboratory, Health and Welfare Canada); these salts of pyrvinium are manufactured no longer. 2-Acetylaminofluorene, pyrvinium pamoate, pyrvinium iodide, and the 6-chloro- and 6-methyl-analogs of pyrvinium iodide were custom-synthesized by Lancaster Synthesis Limited (Eastgate, White Lund, Morecambe, Lancashire, England); this company guarantees the purity of its products to 99.6%.

Monopotassium pamoate, cyclophosphamide, ethidium bromide, 7,12-dimethylbenz[α]anthracene, and 1,2-dimethylhydrazine·2HCl were purchased from the Sigma Chemical Company (Saint Louis, Missouri, U.S.A.).

REAGENTS AND CHEMICALS

All chemicals and reagents employed in the experimental procedures were either A.C.S. or reagent grade in quality, unless specified otherwise. Double-distilled, deionized water (Milli Q® water purification system from Millipore) was used in the preparation of all media, buffers, and solutions.

Mechanical top-loading balances (Sartorius) were used for weighing large quantities of ingredients in the preparation of buffers and media; electronic top-loading analytical balances (Mettler AC100 and AE163) were used for weighing small quantities of amino acids, drugs, and reference standards.

PROCEDURES

A. Analytical Methods

Monopotassium pamoate, pyrvinium chloride, pyrvinium iodide, the USP reference standard of pyrvinium pamoate, and a light-degraded sample of pyrvinium pamoate were examined for purity by TLC and HPLC.

1. Thin-Layer Chromatography

The USP reference standard of pyrvinium pamoate, medical grades I and II (each from a different manufacturer) of pyrvinium pamoate, a sample of medical grade I of pyrvinium pamoate (dissolved in methanol) that had been exposed to room and window light for 9 months, and monopotassium pamoate were analyzed by the TLC method developed by Beckstead and Smith (1967).

a. Qualitative Thin-Layer Chromatography

Eastman Chromagram Sheets were spotted with 10-µl aliquots of each sample solution, the spots not exceeding 2 mm in diameter; each sample had been dissolved in methoxyethanol to a final concentration of approximately 1 mg/ml. These chromatographic sheets (20 cm x 20 cm) are precoated with silica gel adsorbent containing a fluorescent indicator. The adsorbent coating of 100-µm thickness is on an inert, flexible poly(ethyleneterephthalate) support of 200-µm thickness; polyacrylic acid has been added as a binder and the fluorescent indicator is lead-manganese-activated calcium silicate.

The chromatographic sheets were developed, in the dark, for 2 hours in an Eastman Chromagram Developing Apparatus that

contained chloroform:ethanol (7:3) as the developing solvent system. The developed chromatographic sheets were air-dried and examined under long-wave (354-nm), ultraviolet light; the visualized spots were marked and their R_f values were calculated by the conventional formula

$$R_f = \frac{\text{distance traveled by solute}}{\text{distance traveled by solvent}} .$$

b. Preparative Thin-Layer Chromatography

Glass plates (20 cm x 20 cm) were coated with a layer of silica gel of 500-μm thickness (a slurry of 50 g silica gel G, DF-5 [Camag] mixed with 90-100 ml of distilled water). The TLC plates were air-dried and activated at 100°C for 1 hour before use. A 2-ml volume of a stock solution of medical grade I of pyrvinium pamoate (1 mg/ml of methoxyethanol) was applied, in a 5-mm bandwidth, about 2 cm from the bottom of each TLC plate.

The plates were developed, in the dark, for 4-5 hours in chloroform:ethanol (7:3). Preparative TLC plates of 500-μm thickness that have been loaded with such a large quantity of sample require more time to separate than TLC plates of 100-μm thickness that have been spotted with 10 μl of sample. The separation in the preparative TLC was not as well-defined as in the qualitative TLC plates and, therefore, tailing and contamination among the various sections was expected.

The developed, preparative TLC plates were divided into sections corresponding to the separations observed in the qualitative TLC procedure; the components in these sections of silica gel were extracted with methanol and the supernatants were

concentrated by the removal of the solvent. The residues were reconstituted in 15 ml of methanol and centrifuged (Servall refrigerated-automatic centrifuge) for 10 minutes at 15°C but this resulted in the retention of the pyrvinium pamoate by the silica gel. The high affinity of this drug for the silica gel made it difficult to extract this layer further, yet a speed of at least 1500 rpm was required to ensure an extract solution free of silica gel. Qualitative TLC analyses of the methanol extracts confirmed the expected contamination among the various sections of the preparative TLC plates.

2. High Pressure Liquid Chromatography

The high affinity of pyrvinium pamoate for silica gel precluded the use of a normal-phase chromatographic column in the HPLC studies. In reversed-phase TLC, the silica gel is coated with dichloromethylsilane and, with this modification, the R_f values of nonpolar compounds will increase as the lipophilicity of the solvent is increased; with the use of a polar solvent, nonpolar compounds are expected to remain at the origin. This principle of reversed-phase TLC is directly transferable to HPLC and, therefore, separations can be achieved using the greater speed and efficiency of HPLC.

Reversed-phase HPLC involves the use of a nonpolar (hydrophobic) stationary phase rather than the polar (hydrophilic) stationary phases utilized in normal-phase HPLC; a hydrocarbon stationary phase is usually employed and the polarity of the mobile phase is chosen according to the particular properties of the compound under investigation. The elution of compounds from a reversed-phase column occurs in the order of

decreasing polarity, with the most polar compound eluting first and continuing in sequence until the least polar substance has been eluted.

In view of the difficulties inherent in the use of silica gel as a stationary phase in the TLC analyses of pyrvinium pamoate, reversed-phase HPLC was the method of choice for the separation of the components of pyrvinium pamoate.

a. Column

The analytical column (μBondapak C_{18}) was 30 cm in length, with an inside diameter of 3.9 mm and an average particle size of 10 μm. This reversed-phase column consisted of a monomolecular layer of organosilane, bonded permanently to a pellicular silica packing.

$$-\overset{|}{\underset{|}{Si}}-OH + -\overset{|}{\underset{|}{Si}}-(CH_2)_{17}CH_3 \rightarrow -\overset{|}{\underset{|}{Si}}-O-\overset{|}{\underset{|}{Si}}-(CH_2)_{17}CH_3$$

SILICA OCTADECYLSILANE μBondapak C_{18}

b. Instrumentation

Reversed-phase HPLC was performed utilizing a high pressure liquid chromatograph (Waters ALC 202/401) equipped with a solvent delivery system (Model-6000A) and an injection system (U6K Universal Injector). The absorbance of the column eluent was measured with a photometric, fixed-wavelength (254 nm) detector and the fluorescent intensity of the column eluent was monitored continuously (Schoeffel FS 970 LC fluorometer); the absorbance and relative fluorescence were recorded (Fisher Recordall Series 5000 recorder with an input range of 1-10 mV).

c. Chromatographic Conditions

The fluorometer was equipped with an entrance filter (Corning 7-59, range 300-480 nm) and a 389-nm emission filter (range 375-405 nm); the excitation wavelength was set at 355 nm and the range and sensitivity were maintained at 1.0 μA and 4.00, respectively. The sensitivity of the UV detector was set at an absorbance of 0.16, full scale (a range of 16). The most efficient chromatographic solvent system was methanol:water (90:10), the solvent flow rate was 0.5 ml/min at an inlet pressure of 2500 psig, and the chart speed was set at 0.5 cm/min.

Monopotassium pamoate and selected samples of pyrvinium pamoate were analyzed by reversed-phase HPLC using various solvents and combinations of solvents. Methanol and acetonitrile provided the best eluting capacity and, due to the limited solubility of pyrvinium pamoate in predominantly aqueous solvents, high percentages of these powerful eluents were employed as solvents in the HPLC procedures. Methanol alone, combinations of methanol and water (90:10, 80:20, 70:30, and 65:35), combinations of methanol and acetonitrile (90:10 and 80:20), acetonitrile alone, and combinations of acetonitrile and water (90:10 and 80:20) were tested to find the most efficient solvent, or combination of solvents, for the separation of the components of pyrvinium pamoate.

Monopotassium pamoate absorbs UV light more readily than pyrvinium at a wavelength of 254 nm and this is certainly expected since the pamoate moiety has a system of conjugation that is more extended than that of the pyrvinium moiety. The structure of pamoic acid consists of two pairs of fused benzene rings that are

joined by a methylene group (Figure 2); benzene has an absorption band at 254 nm (ϵmax 204, in water) (Dyer, 1965). The intense absorption of pamoic acid at 254 nm, which is due to $\pi \rightarrow \pi^*$ transitions, is augmented by the hydroxyl and carboxyl substituents. Although pyrvinium also contains a conjugated π-electron system, it does not absorb significantly at 254 nm. The high intensity and broad range of absorption of the pamoate moiety may overlap with the absorption of the pyrvinium moiety and may also mask impurities in the pyrvinium pamoate samples. This difficulty was compounded by the fixed wavelength of the detector, which does not permit the search for a more ideal wavelength of absorption.

Reversed-phase fluorescence HPLC has been found to be the most suitable method for the detection of impurities in these samples of pyrvinium pamoate.

d. Drugs and Dosage Forms

Monopotassium pamoate, pyrvinium chloride, pyrvinium iodide, the USP reference standard (A) of pyrvinium pamoate, and several medical grades of pyrvinium pamoate (B, C, D, and E) were prepared for HPLC analysis by dissolution in methanol to a final concentration of 1 mg/ml.

One tablet of each of 3 brands of pyrvinium pamoate (B, C, and D) was finely powdered with the aid of a mortar and pestle, dissolved in 15 ml of methanol, agitated manually for 5 minutes, and then allowed to settle. The tablet excipients precipitated on standing and the supernatants were used in the HPLC analyses.

The tablets were not assayed for drug content and the labeled strength of each tablet (75 mg of pyrvinium pamoate) was assumed. For the qualitative HPLC analyses, the concentration of pyrvinium pamoate in each methanol supernatant was estimated to be 5 mg/ml.

B. Bacterial Assays

Fresh cultures of strains TA98 (genotype *hisD3052 rfa ΔuvrB*) and TA100 (genotype *hisG46 rfa ΔuvrB*) of *Salmonella typhimurium* were prepared from frozen permanents that are stored at -70°C. These strains were streaked for single colonies onto nutrient agar plates and incubated (Fisher Econotemp® incubator, Model 30D) at 37°C for 24 hours. Each single colony was inoculated into 5 ml of L-broth and these cultures were incubated at 37°C in a waterbath (Gyrotory Water Bath Shaker, Model G76, New Brunswick Scientific Company, Incorporated) for about 16 hours to attain stationary-phase cells. The methodologies involved in confirming the genotypes of the tester strains, and the mutagenicity assays, are similar to those described by Ames *et al.* (1975), and by Maron and Ames (1983).

1. Checking the Strains

The genetic markers of the bacterial strains were checked routinely for histidine auxotrophy, sensitivity to crystal violet, sensitivity to ultraviolet radiation, and ampicillin resistance.

a. Histidine Requirement

The bacterial cultures grew on minimal-glucose agar (Vogel-Bonner E medium plus dextrose and agar) only if 0.1 ml of a

solution of histidine plus biotin (0.5 mM histidine·HCl-0.5 mM biotin) had been spread onto the plates; this indicated auxotrophy for histidine.

b. Deep Rough Character

Approximately 10^8 bacteria (0.1 ml of the stationary-phase culture) were spread onto nutrient agar and 3 filter paper discs were placed on top. About 10 µl of a solution of crystal violet (1 mg/ml of water) were applied to each disc and the plate was incubated at 37°C for 12 hours. A clear zone of inhibition around each disc indicated the presence of the *rfa* mutation.

c. Presence of R-Factor

This test was carried out in the same manner as the test for the *rfa* mutation, except that 10 µl of a solution of ampicillin (8 mg/ml 0.02 N NaOH) were applied to the filter paper discs and the nutrient agar plate was incubated at 37°C for 12-24 hours. The absence of a zone of inhibition around each disc indicated ampicillin resistance, thus the pKM101 plasmid was present in the strains.

d. Sensitivity to UV Radiation

Each bacterial culture was spread onto nutrient agar (0.1 ml/plate) and half of each plate was irradiated with a germicidal lamp (General Electric G30T8, 30 watt bulb) for about 30 seconds, at a distance of approximately 40 cm. The time of irradiation was determined empirically. The plate was incubated at 37°C for 12-24 hours. Since strains TA98 and TA100 contain the *uvrB* deletion,

growth was observed only on the unirradiated side of the plate.

2. Mutagenicity Assays

Stock solutions (2.5 mg/ml) of cyclophosphamide, 2-acetylaminofluorene, the USP reference standard of pyrvinium pamoate, and each medical grade of pyrvinium pamoate were prepared in dimethylsulfoxide (DMSO); when required, these stock solutions were diluted with DMSO.

Strains TA98 and TA100 of *Salmonella typhimurium* were cultured in L-broth at 37°C for 16 hours to attain stationary-phase cells. Each treatment tube contained 0.5 ml of S9 mix or 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4), 0.1 ml of stationary-phase bacterial culture (approximately 10^8 bacteria), 0.1 ml of drug solution or 0.1 ml of DMSO, and 2.0 ml of molten top agar that contained 0.2 ml of a solution of 0.5 mM histidine·HCl-0.5 mM biotin. The contents of each tube were poured onto minimal-glucose agar plates (Vogel-Bonner E medium plus dextrose and agar); each assay was performed in quadruplicate. Once the top agar had solidified, the plates were incubated at 37°C; after 48 hours of total incubation time, the *his*$^+$ revertants were scored.

3. Media, Buffers, and Solutions

L-Broth (1.2% tryptone, 1.0% NaCl, 0.5% yeast extract, and 0.1% dextrose), soft agar (0.6% agar and 0.5% NaCl), nutrient agar (0.8% Difco-Bacto nutrient broth, 0.5% NaCl, and 1.5% agar), and minimal-glucose agar (Vogel-Bonner E medium [0.030% $MgSO_4 \cdot 7H_2O$, 0.300% $HOC(CH_2COOH)_2COOH \cdot H_2O$, 1.500% K_2HPO_4, and 0.525% $NaNH_4HPO_4 \cdot 4H_2O$] plus 2.0% dextrose and 1.5% agar) were prepared with double-distilled, deionized water and sterilized (Amsco steam-powered autoclave) at 121°C for 20 minutes. In the preparation of the various media, the solutions

of dextrose were autoclaved separately and added to the media while hot. Similarly, the solutions of the Vogel-Bonner salts (Vogel and Bonner, 1956) and the agar were sterilized separately in the preparation of the minimal-glucose medium.

The sodium phosphate buffer (pH 7.4) consisted of 6.0 ml of 0.1 M monosodium phosphate (1.38 g $NaH_2PO_4 \cdot H_2O$/100 ml water) plus 44.0 ml of 0.1 M disodium phosphate (1.42 g Na_2HPO_4/100 ml water) to make 100 ml of buffer. The salt solution (1.65 M KCl/0.4 M $MgCl_2$) for the S9 mix was prepared by the dissolution of 6.15 g KCl and 4.07 g $MgCl_2 \cdot 6H_2O$ in 50.0 ml of water. The buffer and the salt solution were autoclaved at 121°C for 20 minutes.

The solution of histidine/biotin was prepared by the dissolution of D-biotin (30.9 mg) and L-histidine·HCL (24.0 mg) in 250 ml of sterile water. The histidine/biotin solution and solutions of 0.1 M glucose-6-phosphate (282 mg/10 ml sterile water) and 0.1 M nicotinamide adenine dinucleotide phosphate (38.3 mg NADP/5 ml sterile water) were filter-sterilized and stored at -20°C.

Each ml of S9 mix contained 0.10 ml of rat liver homogenate (obtained from the British Columbia Cancer Foundation, Carcinogen Testing Laboratory), 0.03 ml of 0.1 M NADP, 0.03 ml of 0.1 M glucose-6-phosphate, 0.02 ml of salt solution (1.65 M KCl/0.4 M $MgCl_2$), and 0.82 ml of sodium phosphate buffer (pH 7.4).

C. Yeast Assays

The genotypes of the haploid and diploid strains of *Saccharomyces cerevisiae* that were used in the mutagenicity assays are listed in Table 1. Stocks of these strains were stored, at 4°C, on YEPD-agar (1.0% yeast extract, 2.0% peptone, 2.0% dextrose, and 1.5% agar) supplemented with extra amino acids plus adenine and uracil (L-arginine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-tryptophan, L-valine, adenine, and uracil, each at 20 mg/l; L-leucine at 30 mg/l; and L-threonine at 350 mg/l).

1. Haploid Reversion Assay

Strains XV185-14C, XY718-1A, and 7854-1A of *Saccharomyces cerevisiae* were streaked for single colonies onto YEPD-agar and incubated at 30°C for 48 hours. A single colony of each strain (specifically a pink colony for strain XV185-14C) was selected, streaked onto YEPD-agar supplemented with extra amino acids plus adenine and uracil, and incubated at 30°C for 48 hours.

A cell suspension of each yeast strain was prepared in 5 ml of liquid YEPD medium and the cell concentration of a 100-fold dilution (0.1 ml of cell suspension plus 9.9 ml of buffer or water) was estimated by counting the actual number of cells on a hemocytometer (all 25 squares of the grid) under a light microscope (Leitz Wetzlar 632717, Germany; supplied in Canada by Walter A. Carveth Limited, Scientific Instruments and Photographic Equipment). The cell suspensions were adjusted with liquid YEPD to achieve inocula of 5×10^8 cells/ml for stationary-phase cultures and 5×10^6 cells/ml for logarithmic-phase cultures; 1.0 ml of an adjusted cell suspension was

inoculated into 49.0 ml of a culture medium that consisted of 46.5 ml of liquid YEPD supplemented with 2.5 ml of a stock solution of amino acids plus adenine (L-arginine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-tryptophan, L-valine, and adenine, each at 2 mg/ml; L-leucine at 3 mg/ml; and L-threonine at 30 mg/ml) to achieve a final concentration of $1x10^7$ cells/ml for stationary-phase cultures and $1x10^5$ cells/ml for logarithmic-phase cultures. The cultures, contained in 250-ml Erlenmeyer flasks, were incubated for 15-17 hours in a water-bath shaker (American Optical Corporation, Scientific Instrument Division) at 30°C to obtain logarithmic- or stationary-phase cells.

The cells were harvested by centrifuging (Damon/IEC Division clinical centrifuge) each culture at full speed for 10 minutes; the supernatant (liquid YEPD) was discarded, the pellet of cells was reconstituted in 50 ml of 0.1 M sodium phosphate buffer (pH 7.0), and the cells were washed by centrifuging for another 10 minutes at full speed. The supernatant (buffer) was discarded, the pellet of cells was resuspended in 50 ml of buffer, the cell concentration of a 100-fold dilution (0.1 ml of cell suspension plus 9.9 ml of buffer or water) was estimated by counting the actual number of cells on 25 squares of a hemocytometer grid under a light microscope (this also enabled the discernment between logarithmic and stationary phases of growth by the percentage of budding cells), and the buffered cell suspension was adjusted to $5x10^7$ - $1x10^8$ cells/ml (or to $2x10^8$ cells/ml when toxic compounds were to be analyzed) with 0.1 M sodium phosphate buffer (pH 7.0).

The 0.1 M sodium phosphate buffer (pH 7.0) was prepared by combining 39.0 ml of a solution of 0.2 M monosodium phosphate (27.80 g

$NaH_2PO_4 \cdot H_2O$/1000 ml water) and 61.0 ml of a solution of 0.2 M disodium phosphate (53.65 g $Na_2HPO_4 \cdot 7H_2O$/1000 ml water), and diluting to a total volume of 200.0 ml, as described by Gomori (1955).

Glass test tubes (18 mm x 150 mm), which already contained the substances to be tested (0.1 ml of sterile water, 0.1 ml of DMSO, 0.1 ml of a stock solution of a drug dissolved in DMSO, or crystalline drug plus 0.1 ml of DMSO), each received 3.9 ml of the buffered cell suspension. The tubes were capped, sealed with laboratory film (Parafilm "M"®, American Can Company), and incubated at 30°C in a waterbath shaker; cell samples (0.9 ml for each of 4 sampling times or 1.2 ml for each of 3 sampling times) were taken at the appropriate time intervals and added to plastic test tubes (12 mm x 75 mm) which contained enough 0.1 M sodium phosphate buffer (pH 7.0) to make a total volume of 4.0 ml for strain XV185-14C and 4.5 ml for strains XY718-1A and 7854-1A. The plastic tubes were centrifuged (Damon/IEC Division HN-SII centrifuge) at full speed for 15 minutes to wash the cells; 3.5 ml (strain XV185-14C) or 4.0 ml (strains XY718-1A and 7854-1A) of the supernatant were removed with a 5-ml pipette and the cells were resuspended with the addition of 3.5 ml or 4.0 ml of buffer. This washing procedure was repeated and the final volumes of these cell suspensions (designated as D_0) were 4.0 ml for strain XV185-14C and 4.5 ml for strains XY718-1A and 7854-1A.

Serial dilutions (one 100-fold, two 10-fold, and one 4-fold) were prepared in buffer and 0.2 ml of the appropriate dilution was spread onto each of 4 plates of YEPD-agar or Mortimer complete (MC) medium for the survival estimation. MC medium consists of minimal medium (0.67% Difco yeast nitrogen base without amino acids, 2.00% dextrose,

and 2.00% agar) supplemented with selected amino acids plus adenine and uracil (L-arginine, L-histidine, L-lysine, L-methionine, L-tryptophan, adenine, and uracil, each at 20 mg/l; L-leucine at 30 mg/l; and L-threonine at 350 mg/l). In the preparation of all the media used in the yeast assays, the solutions of dextrose and the solutions of the amino acids plus adenine and uracil were autoclaved separately at 121°C for 20 minutes and added to the media while hot; the solution of L-threonine, which is heat-labile, was filter-sterilized.

Undiluted aliquots of D_0 were spread onto omission media as follows: 0.2 ml/plate on 8 plates of MC minus threonine and on 4 plates each of MC minus arginine, MC minus histidine, and MC minus tryptophan for strain XV185-14C; 0.5 ml/plate on 4 plates each of MC minus histidine and MC minus leucine for strains XY718-1A and 7854-1A. The plates were incubated at 30°C for 4-6 days (strain XV185-14C) and 10-14 days (strains XY718-1A and 7854-1A).

a. Nonsense Suppression

This analysis was carried out with strain XV185-14C by spreading aliquots of the D_0 suspension (0.5 ml/plate) onto 5 plates of MC minus arginine medium with limiting adenine (5 µg/ml). The plates were incubated at 30°C for 4-6 days and pink revertants were recorded as events at the *arg4* locus, whereas white revertants were considered to be events at the nonsense suppressor loci.

b. Coreversion Analysis

The *HIS*$^+$ and *LEU*$^+$ revertants obtained with strains XY718-1A and 7854-1A were patched onto MC minus histidine and MC minus

leucine plates, respectively; the plates were incubated at 30°C for 4 days. These *HIS*$^+$ and *LEU*$^+$ revertants were replica-plated onto MC minus leucine and MC minus histidine media, respectively, and incubated for another 4 days. Corevertants (His$^+$Leu$^+$ phenotype) were recorded as events at the frameshift suppressor loci, whereas single revertants (His$^+$Leu$^-$ or His$^-$Leu$^+$ phenotypes) were considered to be reversion events at the *his4* or *leu2* loci.

2. Diploid Intragenic and Intergenic Mitotic Recombination Assays

Strains D5 and D7 of *Saccharomyces cerevisiae* were streaked for single colonies onto YEPD-agar and incubated at 30°C for 48 hours. A single colony of each strain was selected, streaked onto YEPD-agar (strain D5) or YEPD-agar supplemented with extra amino acids (isoleucine and valine are required) plus adenine and uracil (strain D7), and incubated at 30°C for 48 hours.

The procedures for these assays were similar to those described for the haploid strains except that the cell suspensions (in liquid YEPD medium) were adjusted to $5x10^7$ cells/ml for stationary-phase cultures and $5x10^5$ cells/ml for logarithmic-phase cultures; 1.0 ml of an adjusted cell suspension of strain D5 was inoculated into 49.0 ml of liquid YEPD medium and 1.0 ml of an adjusted cell suspension of strain D7 was inoculated into 49.0 ml of a culture medium that consisted of 46.5 ml of liquid YEPD medium supplemented with 2.5 ml of a stock solution of amino acids (isoleucine and valine are required) plus adenine, to achieve a final concentration of $1x10^6$ cells/ml for stationary-phase cultures and $1x10^4$ cells/ml for logarithmic-phase cultures. These cultures were incubated for 15-17 hours in a waterbath

shaker at 30°C to obtain logarithmic- or stationary-phase cells.

The cells were harvested, washed, and resuspended in 50 ml of buffer; the buffered cell suspensions were adjusted to $5x10^7$ - $1x10^8$ cells/ml (or to $2x10^8$ cells/ml for the analysis of toxic compounds) with 0.1 M sodium phosphate buffer (pH 7.0). The buffered cell suspensions were added (3.9 ml/tube) to the control and treatment tubes, which contained 0.1 ml of DMSO and 0.1 ml of a stock solution of a drug (dissolved in DMSO), respectively; these reaction mixtures were incubated at 30°C in a waterbath shaker. Generally, the procedures for these assays were similar to those described for the haploid strains except that the D_0 suspensions of strain D5 (4.0 ml in volume) were transferred directly into the dilution series without being washed.

a. Intragenic Mitotic Recombination

The D_0 suspensions of strain D7 (also 4.0 ml in volume) were washed twice in buffer and undiluted aliquots were spread (0.3 ml/plate) onto an omission medium (MC minus tryptophan plus isoleucine and valine, each at 20 mg/l); the plates were incubated at 30°C for 4-6 days.

b. Intergenic Mitotic Recombination

Dilution series for strains D5 and D7 were prepared in buffer and the appropriate dilution was spread (0.2 ml/plate) onto 10 plates of MC medium with limiting adenine (5 µg/ml) plus isoleucine and valine (each at 20 µg/ml), or onto YEPD-agar, to obtain 100-200 colonies/plate; YEPD-agar contains a low concentration of adenine, which permits the red and pink color development of adenine-dependent colonies. The plates were incubated at 30°C

for 4 days and then stored at room temperature for at least 6 days to allow time for an adequate color development before the colonies were scored.

3. Induction of Petites in Growing Cells

Strains D5 and N123 of *Saccharomyces cerevisiae* were streaked for single colonies onto YEPG-agar (contains 2.0% glycerol as the carbon source instead of the 2.0% dextrose used in YEPD-agar) and incubated at 30°C for 48 hours; a single colony of each strain was selected, streaked onto YEPG-agar, and incubated at 30°C for 48 hours. A cell suspension of each yeast strain was prepared in 5 ml of liquid YEPG medium (contains 4.0% glycerol) and inoculated into liquid YEPG medium to achieve 50-ml cultures of $1x10^4$ - $1x10^5$ cells/ml for strain D5 and $1x10^5$ - $4x10^5$ cells/ml for strain N123; these cultures were incubated for 16 hours in a waterbath shaker at 30°C. The cultures were centrifuged for 10 minutes, the supernatant (liquid YEPG) was discarded, and the pellet of cells was resuspended in 50 ml of liquid YEPD. The cell suspensions were adjusted to $1x10^7$ cells/ml with liquid YEPD and added (3.9 ml/tube) to the control and treatment tubes, which already contained the substances to be tested. Ethidium bromide was the positive control (Slonimski *et al.*, 1968).

The tubes were incubated at 30°C in a waterbath shaker; cell samples (0.1 ml for strain D5 and 0.8 ml for strain N123) were taken at the appropriate time intervals (0, 0.5, 1, 2, and 3 hours for strain D5 and 1, 2, 4, and 8 hours for strain N123) and added directly to the dilution series. The dilution series were prepared in 0.1 M sodium phosphate buffer (pH 7.0) and each appropriate dilution was spread (0.2 ml/plate) onto regular YEPD-agar (contains 2.0% dextrose) and

onto fortified YEPD-agar (contains 10.0% dextrose). The plates were incubated at 30°C for 3-4 days and the proportion of petite colonies was determined by the tetrazolium-overlay method (Ogur *et al.*, 1957), which involved pouring about 20 ml of tetrazolium top agar (0.1% 2,3,5-triphenyltetrazolium chloride plus 1.5% agar, each dissolved in 0.1 M sodium phosphate buffer at pH 7.0) over the plates; the colonies were examined for color changes at 3, 6, and 24 hours after the overlay procedure.

The plates of strain D5 were not subjected to the overlay procedure until an adequate color development of the adenine-dependent colonies had taken place; since these red and pink colonies could not be scored as petite or grande by the overlay method, they were sampled with a sterile toothpick, patched onto YEPD-agar, and replica-plated onto YEPG-agar.

D. *In Vivo* Mammalian Assays

The genetic activity of pyrvinium pamoate was assessed by the level of chromosomal damage caused in the erythropoietic tissue and colon epithelium of mice. The experimental procedures and the care and treatment of the animals used were in accordance with the principles outlined by the Canadian Council on Animal Care (1980).

1. Induction of Micronuclei in Mouse Bone Marrow

The $LD_{50/7}$ (the dose that kills 50% of the animals within 7 days) of pyrvinium pamoate (dissolved in DMSO) was estimated for the intraperitoneal route of injection in ♀B6C3F1 mice (The Jackson Laboratory, Bar Harbor, Maine, U.S.A.). This established the dose range (0.8 mg/kg was the highest dose that permitted 24 hours of survival) and the animals were treated accordingly.

The mice were weighed (Ohaus triple-beam animal balance) in groups of 5 and the average weight of each mouse (20 g) was used to determine the dosage schedule. The solutions of pyrvinium pamoate (Parke-Davis Canada Incorporated, lot C434662), 1,2-dimethylhydrazine·2HCl, and 7,12-dimethylbenz[a]anthracene were prepared in DMSO and transferred to amber serum vials; the vials were topped with flange-type rubber stoppers and secured with aluminum seals that were applied with a hand-operated crimper. The drugs were injected (Becton-Dickinson 26-gauge Yale® needles, with intradermal bevel, attached to Plastipak® 1-ml tuberculin syringes) intraperitoneally (0.1 ml/mouse). The negative controls were DMSO-treated mice (0.1 ml/mouse) and untreated mice.

The animals were sacrificed by cervical dislocation, carbon dioxide (CO_2) gas, or exposure to CO_2 vapors from dry ice. Bone marrow samples were taken at 24, 48, and 72 hours after the intraperitoneal injections; a femur was removed from each mouse, a small piece of the iliac end of the bone was cut with a pair of scissors, and a pin (about 2.5 cm in length) was inserted into the marrow canal from the epiphyseal end of the femur to push the exudate from the canal. Each bone marrow sample was placed onto a microscope slide, mixed with a drop of fetal calf serum (sterile, newborn-bovine serum from Flow Laboratories), and the preparation was spread across each slide; the slides were allowed to air-dry overnight.

The slides were fixed in absolute methanol for 5 minutes, air-dried for 20 minutes to remove the methanol, and stained in 5% Giemsa (5 ml of Giemsa's stain/100 ml of 0.01 M phosphate buffer [0.71 g Na_2HPO_4 plus 0.68 g KH_2PO_4/1000 ml water] at pH 6.8) for 20 minutes. Giemsa's stain (BDH Chemicals) contains 6.65 g of Giemsa dyes (about 5.0 g azure II-

eosin and 1.6 g azure II), 1.75 ml glycerol, and 823 ml methanol per liter of the commercial dye preparation. The stained slides were rinsed once in the phosphate buffer for 30 seconds and once in distilled water for 5 seconds, air-dried for 1 hour, and the coverslips were mounted with a neutral mounting preparation (DPX® mountant from BDH Chemicals). The details of the slide preparation and staining procedures have been described by Heddle *et al.* (1984).

A light microscope (Leitz Wetzlar 721770, Germany; supplied in Canada by Walter A. Carveth Limited, Scientific Instruments and Photographic Equipment) with oil-immersion objectives was used to score the slides. As a result of the Giemsa stain, white blood cells were deep blue, red blood cells were pink to pale red, polychromatic erythrocytes were light blue and tinged with purple, and micronuclei were deep purple spots that could be identified easily.

2. Induction of Nuclear Aberrations in Mouse Colon

The ♂C57BL mice (The Jackson Laboratory, Bar Harbor, Maine, U.S.A.) were weighed in groups of 5 and the average weight of each mouse (25 g) was used to determine the dosage schedule. The solutions of pyrvinium pamoate (Parke-Davis Canada Incorporated, lot C434662) and 1,2-dimethylhydrazine·2HCl were prepared in DMSO; the dosage forms of pyrvinium pamoate (Vanquin® oral suspension from Parke-Davis Canada Incorporated and Pyr-Pam® oral suspension from ICN Canada Limited) were used as supplied by the manufacturers.

Accurate doses of the drugs were administered to the mice, which had been denied access to food and water for 4 hours prior to dosing, by oral intubation (0.2 ml/mouse) with Perfektum® (Popper & Sons, Incorporated) animal feeding needles (22-gauge, 1-inch long, straight

cannula with a ball diameter of 1.25 mm). The negative controls were DMSO-treated mice (0.2 ml/mouse) and untreated mice.

The animals were sacrificed by cervical dislocation 24 hours after the treatment, and their colons were excised and flushed with phosphate-buffered saline (2.7 mM KCl, 137.0 mM NaCl, 10.0 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) at pH 7.2. Each colon was cut open from the cecum to the anus and rolled from the proximal to the distal end; the rolls of tissue were pinned onto corkboard and fixed for 20 hours in Formalde-Fresh® (Fisher Scientific), which consists of 10% buffered formalin phosphate (pH 6.9 - 7.1) prepared with 4% w/v formaldehyde, 0.4% w/v $NaH_2PO_4 \cdot H_2O$, 0.65% w/v Na_2HPO_4, and 1.5% w/v methanol. The tissue samples were then rinsed and stored in 70% ethanol. The dehydration of the tissue specimens was achieved with one 2-hour bath in 80% ethanol, one 2-hour bath in 90% ethanol, two 1-hour baths in absolute ethanol, and one bath overnight in absolute ethanol.

The dehydrant was cleared from the tissue specimens with one 2-hour, one 4-hour, and one overnight bath in the clearing solution Hemo-De® (Fisher Scientific), which contains terpene, mineral oil, and butylated hydroxyanisole. Paraplast® (Fisher Scientific) tissue embedding medium (pellets of purified paraffin and plastic polymers of regulated molecular weight) was prepared in a vacuum infiltrator/paraffin dispenser (Lipshaw Model 224) set at 56°C. The tissue specimens were transferred from Hemo-De® to stainless-steel tissue capsules, immersed in the liquefied embedding medium, and held in a Napco paraffin oven (Model 5850, National Appliance Company) at 70°C. The embedding procedure involved three 2-hour immersions, plus one overnight immersion, in the liquefied Paraplast®; fresh embedding medium was used at each stage.

The tissue samples were removed from the tissue capsules and each colon specimen was placed into a stainless-steel mold; the molds were filled with liquefied embedding medium from the paraffin dispenser/ vacuum infiltrator and allowed to solidify into blocks. Histologic sections of 6-μm thickness were cut from the paraffin-embedded tissue with a Spencer "820" microtome and deposited on the surface of the fluid (water plus gelatin) in a flotation bath (Lipshaw electric Tissue Float, Model 375) maintained at the lowest temperature setting; this flotation flattened the sections and eliminated the wrinkles. The sections were placed onto microscope slides and prepared for the staining procedure by a succession of dewaxing and hydration steps. The sections were dewaxed in Hemo-De® (two 1-minute baths with agitation) and hydrated in absolute ethanol for 2 minutes, in 95% ethanol for 1 minute, in 70% ethanol for 1 minute, and in distilled water for 3 minutes, with agitation at 30-second intervals in each solvent.

The Feulgen method for staining the sections involved the immersion of the dewaxed and hydrated tissue sections in 5 N HCl, which had been preheated to 50°C, for 2 minutes; the slides were rinsed in two changes of distilled water and immersed in Schiff reagent (Fisher Scientific) for 30 minutes. The commercial preparation of Schiff reagent contains basic fuchsin (pararosaniline·HCl), sodium metabisulfite, and hydrochloric acid; this reagent stains the nuclear chromatin and chromosomes purple. The procedure used in staining the slides was an adaptation of the protocol for the Feulgen method described by Kiernan (1981).

The slides were rinsed in several changes of distilled water for 2-3 minutes and counterstained for 3 minutes with aqueous 0.5% fast green FCF. The stained tissue sections were immersed in distilled

water for 3 minutes, in 70% ethanol for 1 minute, in 95% ethanol for 1 minute, and in absolute ethanol for 2 minutes; the dehydration procedure was completed with two 1-minute baths, with agitation, in Hemo-De®. Finally, the coverslips were mounted with DPX® mountant and a light microscope with oil-immersion objectives was used to examine the tissue sections for nuclear aberrations.

TABLE 1

The strains of *Saccharomyces cerevisiae* used for assaying mutagens and carcinogens

Strain	Genotype	Genetic Endpoints	Reference
XV185-14C	a *trp5-48 arg4-17 lys1-1 ade2-1 his1-7 hom3-10*	Reversion of nonsense (ocher), missense, and putative frameshift mutations	Quah and von Borstel; cited in Shahin and von Borstel (1977, 1978)
XY718-1A	a *his4-519 leu2-3*	Reversion of frameshift mutations: locus and suppressors	Culbertson *et al.* (1977, 1980)
7854-1A	a *his4-38 leu2-3*	Reversion of frameshift mutations: locus and suppressors	Culbertson *et al.* (1977, 1980)
N123	a *his1* (*rho*$^{+}$ *ome*$^{-}$ *CHL*^{S} *ERY*^{S} *OLI*^{S})	Induced *rho*$^{-}$ phenotype, mutations in mitochondrial genome detected by resistance to antibiotics (chloramphenicol, erythromycin, and oligomycin)	Moustacchi *et al.* (1976) Juliani *et al.* (1976)
D5	$\frac{\alpha}{a}$ $\frac{trp1\text{-}1}{+}$ $\frac{ade2\text{-}40}{ade2\text{-}119}$ $\frac{MAL1}{+}$ $\frac{+}{MAL4}$	Mitotic recombination, gene conversion, and mutation	Zimmermann (1973)
D7	$\frac{\alpha}{a}$ $\frac{ade2\text{-}40}{ade2\text{-}119}$ $\frac{trp5\text{-}12}{trp5\text{-}27}$ $\frac{ilv1\text{-}92}{ilv1\text{-}92}$	Intragenic recombination, induced mitotic recombination, gene conversion, and reverse mutations	Zimmermann *et al.* (1975)

RESULTS AND DISCUSSION

ARTICLE I

The Genetic Activity in *Saccharomyces cerevisiae* and Thin-layer Chromatographic Comparisons of Medical Grades of Pyrvinium Pamoate and Monopyrvinium Salts

Excerpts of Article I have been published

by

R.D. Mehta, U.G.G. Hennig, R.C. von Borstel, and L.G. Chatten
in *Mutation Research* **102**: 59-69 (1982).

ABSTRACT

The pamoate, chloride, and iodide salts of pyrvinium, which is a cyanine dye with anthelmintic properties, were studied in a diploid mitotic recombination and gene conversion assay (strain D5 of *Saccharomyces cerevisiae*) and in a haploid yeast reversion assay (strain XV185-14C). With the use of a thin-layer chromatographic (TLC) detection technique, samples of pyrvinium pamoate from several sources were found to contain different numbers and quantities of impurities. All of the samples of pyrvinium pamoate and the monopyrvinium salts were recombinogenic in strain D5 and mutagenic in strain XV185-14C, and the degree of genetic activity varied among the medical grades of pyrvinium pamoate that were tested. Monopotassium pamoate was found to be inactive genetically in both yeast strains. Light-catalyzed degradation did not enhance the genetic activity of pyrvinium pamoate in either of the yeast strains; the degraded samples were not mutagenic.

INTRODUCTION

The cyanine dye pyrvinium chloride was observed to be highly effective against the natural pinworm (*Enterobius* [*Oxyuris*] *vermicularis*) infestations in mice and rats (Weston *et al.*, 1953), and in the treatment of oxyuriasis in humans (Sawitz and Karpinski, 1956). The chloride salt of pyrvinium was not well-tolerated as it caused gastrointestinal upset and renal damage that was believed to be reversible upon the withdrawal of the therapeutic agent (Hales and Welch, 1953).

Pyrvinium chloride was eventually replaced by the equally effective pyrvinium pamoate, which produces less gastrointestinal upset and is not absorbed systemically (Rollo, 1980).

Pyrvinium pamoate (Figure 2) has been shown to be mutagenic in the *Salmonella typhimurium* microsomal test system (MacPhee and Podger, 1977; Espinosa *et al.*, 1981; Lake and de la Iglesia, 1981). The drug was also found to be active genetically in a haploid yeast reversion assay (Galindo, 1979; Galindo *et al.*, 1979; Hennig *et al.*, 1981) and in a diploid mitotic recombination and gene conversion assay (Hennig *et al.*, 1981), in both logarithmic- and stationary-phase cells.

In this investigation two medical grades of pyrvinium pamoate, which fulfilled the specifications of the "United States Pharmacopeia" (USP XX, 1980), have been compared to the USP reference standard of the drug in the mutagenicity tests.

MATERIALS AND METHODS

TLC Studies

Monopotassium pamoate, the USP reference standard of pyrvinium pamoate (lot F2), medical grades I and II (each from a different manufacturer) of pyrvinium pamoate, and a sample of medical grade I that had been exposed to light were analyzed by the TLC method that was developed by Beckstead and Smith (1967). Each sample was dissolved in methoxyethanol to a final concentration of approximately 1 mg/ml.

For the qualitative TLC analyses, Eastman Chromagram Sheets were spotted with 10-µl aliquots of each sample solution, the spots not

exceeding 2 mm in diameter. The chromatographic sheets were developed, in the dark, for 2 hours in a chloroform:ethanol (7:3) solvent system, air-dried, and examined under long-wave (354-nm) ultraviolet light.

For the preparative TLC procedure, glass plates (20 cm x 20 cm) that were coated with a layer of silica gel (500-μm thickness) were activated at 100°C for one hour before use. A 2-ml volume of a stock solution of medical grade I of pyrvinium pamoate that was dissolved in methoxyethanol (1 mg/ml) was applied, in a 5-mm band width, 2 cm from the bottom of each TLC plate. The plates were developed, in the dark, for 4-5 hours in chloroform:ethanol (7:3). The separation in the preparative TLC plates was not as well-defined as in the qualitative TLC plates and, therefore, tailing and contamination among the various sections were expected.

The developed preparative TLC plates were divided into sections corresponding to the separations that were observed in the qualitative TLC procedure. The silica gel fractions were extracted with methanol and the extracts were concentrated by the removal of the solvent. The concentrated methanol extracts were centrifuged at 1500 rpm for 10 minutes at 15°C but this resulted in the retention of the pyrvinium pamoate by the silica gel. The high affinity of the drug for the silica gel made it difficult to extract this layer further. TLC analyses of the methanol supernatants confirmed the expected contamination among the various sections of the preparative TLC plates. In addition, the three fractions of medical grade I of pyrvinium pamoate that had been separated in the preparative TLC procedure did not exhibit genetic activity in strains D5 and XV185-14C of *Saccharomyces cerevisiae* (Mehta *et al.*, 1981).

Pyrvinium Salts

Stock solutions of monopotassium pamoate (6.2 mg/ml), pyrvinium chloride (18.4 and 13.4 mg/ml), pyrvinium iodide (18.4 and 16.3 mg/ml), and all samples of pyrvinium pamoate (18.4 and 11.1 mg/ml) were prepared in dimethylsulfoxide (DMSO). The solutions were stored in glassware that was wrapped with aluminum foil to prevent photodegradation (Beckstead and Smith, 1967) and when required, the stock solutions were diluted with DMSO. All of these experiments were conducted with a minimum of exposure to visible light.

A crystalline sample of medical grade I of pyrvinium pamoate was exposed to room and window light for a period of two years. A second sample of medical grade I was dissolved in methanol and the solution was exposed to light for nine months; the solvent was removed and the residue was used in all of the testing procedures. Stock solutions (18.4 mg/ml) of the residue and the photodegraded crystalline material were prepared in DMSO.

Yeast Strains

The haploid strain XV185-14C of *Saccharomyces cerevisiae* was used for studying reverse mutations. This strain has the genotype

a *ade2-1 arg4-17 lys1-1 trp5-48 his1-7 hom3-10*;

his1-7 is a missense mutation, *hom3-10* is a putative frameshift mutation, and *ade2-1*, *arg4-17*, *lys1-1*, and *trp5-48* are suppressible ocher nonsense mutations.

The mitotic recombination and gene conversion studies were carried out in the diploid strain D5 of *Saccharomyces cerevisiae* (Zimmermann, 1973). The genotype of this strain is $\frac{\alpha}{a}\ \frac{trp1\text{-}1}{+}\ \frac{ade2\text{-}40}{ade2\text{-}119}\ \frac{MAL1}{+}\ \frac{+}{MAL4}$.

The allele *ade2-40* confers a red coloration to the colonies, whereas the leaky allele *ade2-119* causes the colonies to be pink. When these two alleles of the *ade2* gene are present in the same diploid strain, they complement each other and, as a result, white colonies are formed.

Reversion Assay (Strain XV185-14C)

Cells that had been grown on YEPD-agar plates (yeast extract 1.0%, peptone 2.0%, dextrose 2.0%, and agar 1.5%) for 48 hours were inoculated into liquid YEPD medium to achieve a concentration of $1x10^7$ cells/ml and the culture was incubated for 15-17 hours in a waterbath shaker at 30°C until the cells reached stationary phase. The cells were harvested by centrifugation and washed twice in 0.1 M phosphate buffer (pH 7.0).

For the mutagenicity assays, which have been described previously (von Borstel *et al.*, 1981c), a cell suspension ($5x10^7$ - $1x10^8$ cells/ml) of each culture was prepared in buffer and dispensed into glass test tubes (3.9 ml/tube). A 0.1-ml aliquot of an appropriate dilution (in DMSO) of the stock solution of a drug, or 0.1 ml of DMSO, was added to each tube and the reaction mixtures were incubated at 30°C, in the dark, in a waterbath shaker. Cell samples were taken at predesignated time intervals and washed twice, by centrifugation, in phosphate buffer. Each sample was then resuspended in 4.0 ml of phosphate buffer.

Serial dilutions were made in phosphate buffer, and 0.2 ml of cell suspension from each of the control and treatment tubes was spread onto YEPD-agar plates for the survival estimation. Undiluted samples were spread onto omission media (0.2 ml/plate). The plates were incubated

at 30°C for 4-6 days. The complete medium from which the omission media were derived consisted of 0.67% Difco yeast nitrogen base without amino acids, 2.00% dextrose, 2.00% agar, plus all of the required nutritional supplements. The details for the preparation of these media have been described by von Borstel *et al.* (1971). In the composition of an omission medium the nutritional requirement, histidine in this investigation, for which revertants are sought, is omitted.

Mitotic Recombination and Gene Conversion Assay (Strain D5)

The procedures for this assay were similar to those described for the haploid strain XV185-14C except that the initial inoculum that was used for growing stationary-phase cells was $1x10^6$ cells/ml.

At the termination of a treatment period, cell samples were washed by centrifugation and resuspended in 4.0 ml of phosphate buffer. A dilution series was prepared for each sample and 0.2 ml of cell suspension from each of the control and treatment tubes was spread onto YEPD-agar plates to obtain 100-200 colonies per plate. YEPD medium contains a low concentration of adenine, which permits the red and pink color development of adenine-dependent colonies. The plates were incubated at 30°C for 7-10 days before they were scored.

RESULTS AND DISCUSSION

The TLC plates in Figures 3 and 4 illustrate the separation that was achieved for two side-by-side trials of several medical grades of pyrvinium pamoate, the USP reference standard of pyrvinium pamoate, monopotassium pamoate, and the light-exposed sample of medical grade I of pyrvinium pamoate. Medical grade I separated into three components, whereas the USP reference standard separated into four components. These results were in agreement with those that were obtained by Beckstead and Smith (1967). Medical grade II had seven components and the sample of medical grade I that had been exposed to light had twelve components. The R_f values for the components of the separations that are depicted in Figures 3 and 4 are listed in Tables 2 and 3.

In the TLC analyses, monopotassium pamoate had an R_f value of 0.96 (Table 2 and Figure 3), and this same component was observed in the USP reference standard, medical grade I, medical grade II, and the light-exposed sample of pyrvinium pamoate (Tables 2 and 3; Figures 3 and 4). An intensely red spot with extensive tailing was observed at R_f 0.65 in the USP reference standard and medical grade I of pyrvinium pamoate (Table 2 and Figure 3). This component, which is the pyrvinium moiety, is a major component of pyrvinium pamoate.

It is evident from the chemical structure of pyrvinium pamoate (Figure 2) that the existence of geometric isomers of pyrvinium is possible. The α- and β-hydrogens of the vinyl bond could be in *cis*-configurations or in *trans*-configurations. The results that were obtained with fluorescence high pressure liquid chromatography indicate that one of these isomers is more abundant (Figure 5; this volume,

p. 82), and nuclear magnetic resonance spectroscopy has established that the *trans*-isomer is the more abundant of the geometric isomers of pyrvinium (this volume, p. 129). On this basis, the intensely red component at R_f 0.65 in the USP reference standard and medical grade I of pyrvinium pamoate can be identified tentatively as the *trans*-isomer of pyrvinium.

The less intense components at R_f values of 0.52 and 0.42 in the USP reference standard and medical grade I of pyrvinium pamoate (Table 2 and Figure 3) are probably the less abundant of the geometric isomers of pyrvinium. The USP reference standard of pyrvinium pamoate contains an additional component at R_f 0.26 (Table 2) and, judging from the low intensity of this spot (Figure 3), it may be another isomer of pyrvinium. The existence of these geometric isomers in the USP reference standard and in medical grades of pyrvinium pamoate has been alluded to by Beckstead and Smith (1967).

It is apparent that medical grade II of pyrvinium pamoate contains several impurities, as well as higher relative quantities of the isomers of pyrvinium (Table 3 and Figure 4); in fact, medical grade II probably contains all of the geometric isomers of pyrvinium that are possible. Both the pyrvinium moiety and the pamoate moiety appear to have been degraded in the light-exposed sample of medical grade I of pyrvinium pamoate (Tables 2 and 3; Figures 3 and 4).

Mutagenicity Studies

The data that are reported in Tables 4-8 are for stationary-phase cells of *Saccharomyces cerevisiae*. Similar results, with respect to the genetic activity of the drugs that had been tested, were obtained

with logarithmic-phase cells (von Borstel *et al.*, 1981a).

An enhancement in the cell viability was observed in the presence of pyrvinium pamoate, the monopyrvinium salts, and monopotassium pamoate (Tables 4-8). This is an artefact that was due to the effect of a semisolid drug matrix which precipitated during centrifugation because of the limited aqueous solubility of these drugs. The matrix trapped many of the cells and prevented their removal in the subsequent washing procedure. The control tubes did not exhibit this matrix formation and some of the cells were lost at each washing, thus the apparent enhancement of cell survival is not real.

Most mutagens cause cell death as well as genetic change, and these two effects must be separated if mutation induction is to be quantified. The quantification of mutation induction is expressed as a relative reversion frequency, which is calculated by dividing the number of genetic events that were detected by the number of surviving cells. The concentrations of toxic chemicals are chosen to permit a reasonable survival rate without eliminating a significant mutagenic effect, and this is important because the calculated reversion frequencies may be exaggerated when the survival rate is low. When the solubility limits of a compound are exceeded, a dose-response relationship can be obtained by extending the duration of the treatment.

Compounds that show reversion frequencies or percentages of aberrant colonies that are at least 2-fold higher than those of the negative control are classified as mutagenic, whereas compounds that consistently show less than 1.5 times the reversion frequencies of the negative control are classified as nonmutagenic. When the reversion frequencies are consistently higher (about 1.5-fold) than the negative

control, the compound is classified as weakly mutagenic (von Borstel *et al.*, 1981c). The lowest limit of detection is a doubling of the reversion frequency over the concurrent negative control, which is the vehicle. Reproducibility can be demonstrated by a mutagenic effect in several parallel cultures, or in several independent experiments. The interpretation of the data that is obtained in the testing of chemicals for genetic activity in *Saccharomyces cerevisiae* has been reviewed by Zimmermann *et al.* (1984).

The USP reference standard and medical grades I and II of pyrvinium pamoate induced mitotic recombination and aberrant colonies in strain D5 of *Saccharomyces cerevisiae* (Table 4), and these samples of pyrvinium pamoate increased the frequency of *HIS*$^+$ revertants above the spontaneous level in strain XV185-14C (Table 5). These effects were observed only after 24 hours of exposure to the USP reference standard and medical grade I, whereas a significant increase in genetic activity was observed for medical grade II after shorter (2 hours), as well as longer (24 hours), treatment periods (Tables 4 and 5). In addition, medical grade II was toxic, as is evident from the survival data for strains D5 and XV185-14C (Tables 4 and 5).

In order to determine whether or not the light-catalyzed degradation of pyrvinium pamoate contributed to the enhanced genetic activity and toxicity of medical grade II (see Tables 4 and 5), a sample of medical grade I that was dissolved in methanol and a crystalline sample of medical grade I were exposed to room and window light at room temperature for nine-month and two-year time spans, respectively. The solution gradually darkened in color and once the methanol had evaporated, the color of the residue was indistinguishable from that of

medical grade II. The crystalline sample also darkened in color but not to the same degree as the sample that was dissolved in methanol.

Monopotassium pamoate and the residue of the light-exposed solution of medical grade I of pyrvinium pamoate were not seen to be active genetically in either the diploid (strain D5) or the haploid (strain XV185-14C) yeast assays, even after a 24-hour treatment period (Tables 4 and 5). The light-exposed crystalline sample appears to have induced a 2-fold increase in the frequency of aberrant colonies after 24 hours of exposure (Table 4) and, although this genetic effect is not as strong as that caused by medical grade I after a 24-hour exposure period (Table 4), the results appear to be consistent with the observation that pyrvinium pamoate degrades more rapidly in solution than in crystalline form (Beckstead and Smith, 1967). These data indicate that the light-catalyzed degradation of pyrvinium pamoate is not responsible for the genetic activity that was observed with the USP reference standard and the medical grades of pyrvinium pamoate. In fact, the genetic activity of the drug appeared to decrease gradually as a consequence of photodegradation.

The monopyrvinium salts, pyrvinium chloride and pyrvinium iodide, were found to be highly toxic at concentrations greater than 18.40 µg/ml, therefore lower concentrations and a shorter exposure time (1 hour) were used in these experiments (Tables 6 and 7). At concentrations of 18.40 µg/ml and 3.68 µg/ml, pyrvinium iodide and pyrvinium chloride induced mutations in strain XV185-14C (Table 6) and mitotic recombination and aberrant colonies in strain D5 (Table 7), with frequencies significantly higher than the control values.

Medical grade II of pyrvinium pamoate, at concentrations of 0.74,

3.68, and 18.40 µg/ml, was used as an additional control in these experiments. The relative genetic response to the monopyrvinium salts was significantly stronger than the response to these low doses of medical grade II, which did not increase the frequencies of genetic alterations above the spontaneous level (Tables 6 and 7). Pyrvinium iodide and pyrvinium chloride were more toxic and exhibited stronger genetic activity when they were compared to equimolar concentrations of pyrvinium pamoate (Table 8).

In the comparison of equimolar concentrations of several pyrvinium salts, strain D5 was exposed to twice the concentration of pyrvinium ion when it was treated with pyrvinium pamoate, yet pyrvinium chloride and pyrvinium iodide were more toxic and elicited stronger genetic responses (Table 8). Pyrvinium pamoate, which is not absorbed systemically (Rollo, 1980), is the salt of a weak acid and a weak base, therefore it is not unexpected that this dipyrvinium salt does not dissociate readily at physiological pH values. In contrast, pyrvinium chloride and pyrvinium iodide, which are salts of strong acids and a weak base, ionize to a greater extent. Ionization results in increased solubility and the solubility of a drug at physiological pH affects its availability for absorption and its passage across cell membranes. The monopyrvinium salts are absorbed systemically (Hales and Welch, 1953). The availability for cellular uptake appears to be an important factor in the degree of genetic activity that is exhibited by the various pyrvinium salts.

Pyrvinium salts, including pyrvinium pamoate from all sources, induce mitotic recombination and aberrant colonies in diploid yeast, and they are mutagenic in the haploid yeast reversion test. The

monopyrvinium salts are more toxic and they are stronger mutagens than the USP reference standard and the medical grades of pyrvinium pamoate. The light-catalyzed degradation of pyrvinium pamoate is not the factor that is responsible for the genetic activity of the drug. The enhanced genetic activity and toxicity of medical grade II may be due to one or more of the numerous impurities that were revealed in the TLC analyses. The absence of a mutagenic response to monopotassium pamoate and the strong mutagenic response to the monopyrvinium salts indicate that the pyrvinium moiety itself is involved in the genetic activity of pyrvinium pamoate.

TABLE 2

A summary of the data for the thin-layer analysis of several samples of pyrvinium pamoate and monopotassium pamoate

Sample	Distance Moved by Solute (cm)[a]	R_f Value[b]	Color under 354-nm UV Light
solution of medical grade I of pyrvinium pamoate exposed to light for 9 months	12.2	0.97	light green
	11.9	0.94	orange
	10.5	0.84	orange
	9.4	0.75	orange
	8.2	0.66	orange
	6.7	0.53	light green
	5.4	0.42	bright red
	3.6	0.29	light orange
	3.3	0.26	light green
	2.3	0.18	orange
	0.9	0.07	light green
	0.5	0.04	orange
monopotassium pamoate	12.0	0.96	green
medical grade I of pyrvinium pamoate	11.9	0.95	light green (orange tinge)
	8.2 (tailing from 7.0 - 9.4 cm)	0.65	red
	6.4	0.52	orange
	5.2	0.42	orange

TABLE 2 (continued)

Sample	Distance Moved by Solute (cm)[a]	R_f Value[b]	Color under 354-nm UV Light
USP reference standard of pyrvinium pamoate	12.4	0.98	light green (orange tinge)
	8.3 (tailing from 7.2 - 9.4 cm)	0.65	red
	6.6	0.51	orange
	5.3	0.42	orange
	3.4	0.26	light orange

[a] The solvent system was chloroform:ethanol (7:3) and the solvent front was at 12.6 cm.

[b] Each R_f value is the average of two side-by-side trials, as depicted in Figure 3.

TABLE 3

A summary of the data for the thin-layer analysis of several samples of pyrvinium pamoate

Sample	Distance Moved by Solute (cm)[a]	R_f Value[b]	Color under 354-nm UV Light
solution of medical grade I of pyrvinium pamoate exposed to light for 9 months	14.6	0.97	light green
	13.2	0.88	orange
	11.6	0.78	orange
	10.0	0.66	light green
	8.2	0.54	orange
	6.4	0.43	orange
	5.8	0.39	light green
	4.0	0.26	orange
	2.2	0.15	light green
	1.2	0.08	orange
medical grade II of pyrvinium pamoate	14.5	0.98	light green (orange tinge)
	12.0	0.80	bright red
	10.2	0.68	bright red
	8.9	0.60	bright red
	7.5	0.50	orange
	5.6	0.38	light green
	1.6	0.11	orange
medical grade I of pyrvinium pamoate	14.5	0.95	light green
	12.0	0.78	red
	10.2	0.67	orange
	7.7	0.50	orange

[a] The solvent system was chloroform:ethanol (7:3) and the solvent front was at 15.0 cm.

[b] Each R_f value is the average of two side-by-side trials, as depicted in Figure 4.

TABLE 4

Induction of mitotic recombination and aberrant colonies in strain D5 of *Saccharomyces cerevisiae* after treatment with monopotassium pamoate and several samples of pyrvinium pamoate

Treatment[a]	Exposure Time (hours)	Total Colonies[b]	Survival (%)	Colony Phenotype[c]						Mitotic Recombination[d] (%)	Total Aberrant Colonies[e] (%)
				RP	RPW	R	RW	P	PW		
control (2.5% DMSO)	2	1315	100	1	0	2	0	6	1	0.08	0.76
	24	1093	100	0	0	3	2	2	1	<0.09	0.7
medical grade I	2	1711	152	1	0	3	4	7	1	0.06	0.94
	24	803	74	5	0	3	3	5	3	0.6	2.4
crystalline medical grade I exposed to light	2	1595	142	0	0	6	4	7	1	<0.06	1.1
	24	1273	116	2	0	6	0	7	3	0.2	1.4
control (2.5% DMSO)	2	1217	100	0	0	0	3	0	0	<0.08	0.2
	24	1008	100	0	1	0	0	0	0	0.1	0.1
residue of the solution of medical grade I exposed to light	2	1521	139	0	0	0	1	0	0	<0.07	0.07
	24	1587	144	0	0	0	1	0	1	<0.06	0.1
medical grade II	2	260	0.3	1	3	7	11	4	6	2	12
	24	63	0.006	1	0	3	0	34	0	2	60
USP reference standard	2	1671	153	6	0	1	1	0	0	0.4	0.5
	24	2233	61	7	6	2	5	4	10	0.58	1.5

TABLE 4 (continued)

Treatment[a]	Exposure Time (hours)	Total Colonies[b]	Survival (%)	Colony Phenotype[c]						Mitotic Recombination[d]	Total Aberrant Colonies[e] (%)
				RP	RPW	R	RW	P	PW		
monopotassium pamoate	2	1730	142	0	0	1	1	1	0	<0.06	0.2
	24	2442	140	0	0	0	2	3	0	<0.04	0.2

[a]The doses were 3.6×10^{-4} M for monopotassium pamoate and 4×10^{-4} M for each sample of pyrvinium pamoate. Medical grade I and the light-exposed crystalline sample of medical grade I were tested in a separate experiment.

[b]The total number of surviving colonies on a complete medium (YEPD-agar) that contains a low concentration of adenine.

[c]The abbreviations RP, RPW, R, RW, P, and PW refer to red-pink, red-pink-white, red, red-white, pink, and pink-white colonies, respectively.

[d]Represented by RP (twin-spot) and RPW colonies.

[e]Includes RP, RPW, R, RW, P, and PW colonies.

TABLE 5

Reversion frequencies (*HIS*$^+$) induced in strain XV185-14C of *Saccharomyces cerevisiae* after treatment with monopotassium pamoate and several samples of pyrvinium pamoate

Treatment[a]	Exposure Time (hours)	Survival[b] (%)		Reversion Frequencies[c] (*HIS*$^+$ revertants/10^7 survivors)
control (2.5% DMSO)	2	100	(480)	43 (83)
	24	100	(374)	42 (63)
medical grade I	2	118	(564)	36 (80)
	24	96	(541)	89 (129)
control (2.5% DMSO)	2	100	(443)	48 (213)
	24	100	(659)	36 (237)
medical grade II	2	86	(571)	144 (549)
	24	0.09	(144)	2000 (6)
USP reference standard	2	175	(774)	29 (225)
	24	66	(434)	82 (354)
residue of the solution of medical grade I exposed to light	2	188	(1249)	22 (186)
	24	118	(778)	22 (171)
monopotassium pamoate	2	193	(853)	25 (210)
	24	95	(629)	34 (212)

[a]The doses were 3.6×10^{-4} M for monopotassium pamoate and 4×10^{-4} M for each sample of pyrvinium pamoate. Medical grade I was tested in a separate experiment.

[b]The numbers in parentheses constitute the total number of surviving colonies on 4 plates of YEPD-agar.

[c]The numbers in parentheses constitute the total number of revertant colonies, as determined by plate counts.

TABLE 6

Reversion frequencies (*HIS*$^+$) induced in strain XV185-14C of *Saccharomyces cerevisiae* after 1 hour of exposure to several pyrvinium salts

Treatment[a]	Dose[b] (µg/ml)	Survival[c] (%)	Reversion Frequencies[d] (*HIS*$^+$ revertants/10^7 survivors)
control	--	100 (1276)	71 (454)
pyrvinium pamoate (medical grade II)	0.74	113 (722)	74 (533)
	3.68	117 (744)	72 (537)
	18.40	114 (726)	76 (553)
pyrvinium chloride	0.74	93 (594)	78 (461)
	3.68	95 (605)	196 (564)
	18.40	45 (288)	273 (786)
pyrvinium iodide	0.74	75 (476)	95 (452)
	3.68	91 (582)	114 (665)
	18.40	7 (217)	912 (394)

[a]The control was DMSO (25 µl/ml).

[b]The corresponding molarities for these doses are: 1.6×10^{-5}, 8.0×10^{-5}, and 4.0×10^{-4} for pyrvinium pamoate; 4.4×10^{-5}, 2.2×10^{-4}, and 1.1×10^{-3} for pyrvinium chloride; and 3.6×10^{-5}, 1.8×10^{-4}, and 9.0×10^{-4} for pyrvinium iodide.

[c]The numbers in parentheses constitute the total number of surviving colonies on 4 plates of MC medium.

[d]The numbers in parentheses constitute the total number of revertant colonies, as determined by plate counts.

TABLE 7

Induction of mitotic recombination and aberrant colonies in strain D5 of *Saccharomyces cerevisiae* after 1 hour of exposure to several pyrvinium salts

Treatment[a]	Dose[b] (µg/ml)	Total Colonies[c]	Survival (%)	Colony Phenotype[d]						Mitotic Recombination[e] (%)	Total Aberrant Colonies[f] (%)
				RP	RPW	R	RW	P	PW		
control	--	1646	100	0	1	0	0	1	1	0.06	0.2
pyrvinium pamoate (medical grade II)	0.74	1391	237	0	0	0	0	2	1	<0.07	0.2
	3.68	2879	242	0	0	1	0	0	0	<0.04	0.04
	18.40	2457	200	0	0	0	0	1	3	<0.04	0.2
pyrvinium chloride	0.74	2742	176	0	0	0	2	1	0	<0.04	0.1
	3.68	2849	196	2	1	0	0	1	2	0.1	0.2
	18.40	737	64	2	9	12	20	13	7	1.5	8.6
pyrvinium iodide	0.74	2262	162	0	0	0	0	0	0	<0.04	<0.04
	3.68	1749	157	1	1	6	12	5	15	0.1	2.3
	18.40	174	0.2	3	3	3	17	4	6	3	21

[a] The control was DMSO (25 µl/ml).

[b] The corresponding molarities for these doses are: 1.6×10^{-5}, 8.0×10^{-5}, and 4.0×10^{-4} for pyrvinium pamoate; 4.4×10^{-5}, 2.2×10^{-4}, and 1.1×10^{-3} for pyrvinium chloride; and 3.6×10^{-5}, 1.8×10^{-4}, and 9.0×10^{-4} for pyrvinium iodide.

[c] The total number of surviving colonies on a complete medium containing a limiting concentration of adenine (MC with 5 µg adenine/ml).

[d] The abbreviations RP, RPW, R, RW, P, and PW refer to red-pink, red-pink-white, red, red-white, pink, and pink-white colonies, respectively.

[e] Represented by RP (twin-spot) and RPW colonies.

[f] Includes RP, RPW, R, RW, P, and PW colonies.

TABLE 8.

Induction of mitotic recombination and aberrant colonies in strain D5 of *Saccharomyces cerevisiae* after 2 hours of exposure to equimolar concentrations of several pyrvinium salts

Treatment[a]	Concentration (moles/l)	Pyrvinium[b] (µg/ml)	Total Colonies[c]	Survival (%)	Colony Phenotype[d]						Mitotic Recombination[e] (%)	Total Aberrant Colonies[f] (%)
					RP	RPW	R	RW	P	PW		
control	--	--	2437	100	3	1	1	2	7	4	0.2	0.74
pyrvinium pamoate (medical grade II)	$1x10^{-5}$	7.6	2401	79	6	2	5	5	12	11	0.3	1.7
	$2x10^{-5}$	15.3	2968	97	16	2	6	5	8	8	0.61	1.5
	$4x10^{-5}$	30.6	1729	57	13	0	4	1	10	15	0.75	1.9
pyrvinium chloride	$1x10^{-5}$	3.8	2472	81	18	4	5	9	13	11	0.89	2.4
	$2x10^{-5}$	7.6	2557	46	28	1	7	7	7	12	1.1	2.4
	$4x10^{-5}$	15.3	2176	39	26	2	8	14	25	4	1.3	3.6
pyrvinium iodide	$1x10^{-5}$	3.8	2089	43	11	3	5	6	12	4	0.67	2.0
	$2x10^{-5}$	7.6	1583	30	14	5	8	6	10	11	1.2	3.4
	$4x10^{-5}$	15.3	1250	24	13	1	12	6	10	8	1.1	4.0

[a] The control was DMSO (25 µl/ml).

[b] Equivalent weight of pyrvinium ion, calculated for the listed molarities of each of the pyrvinium salts, to which the cells were exposed.

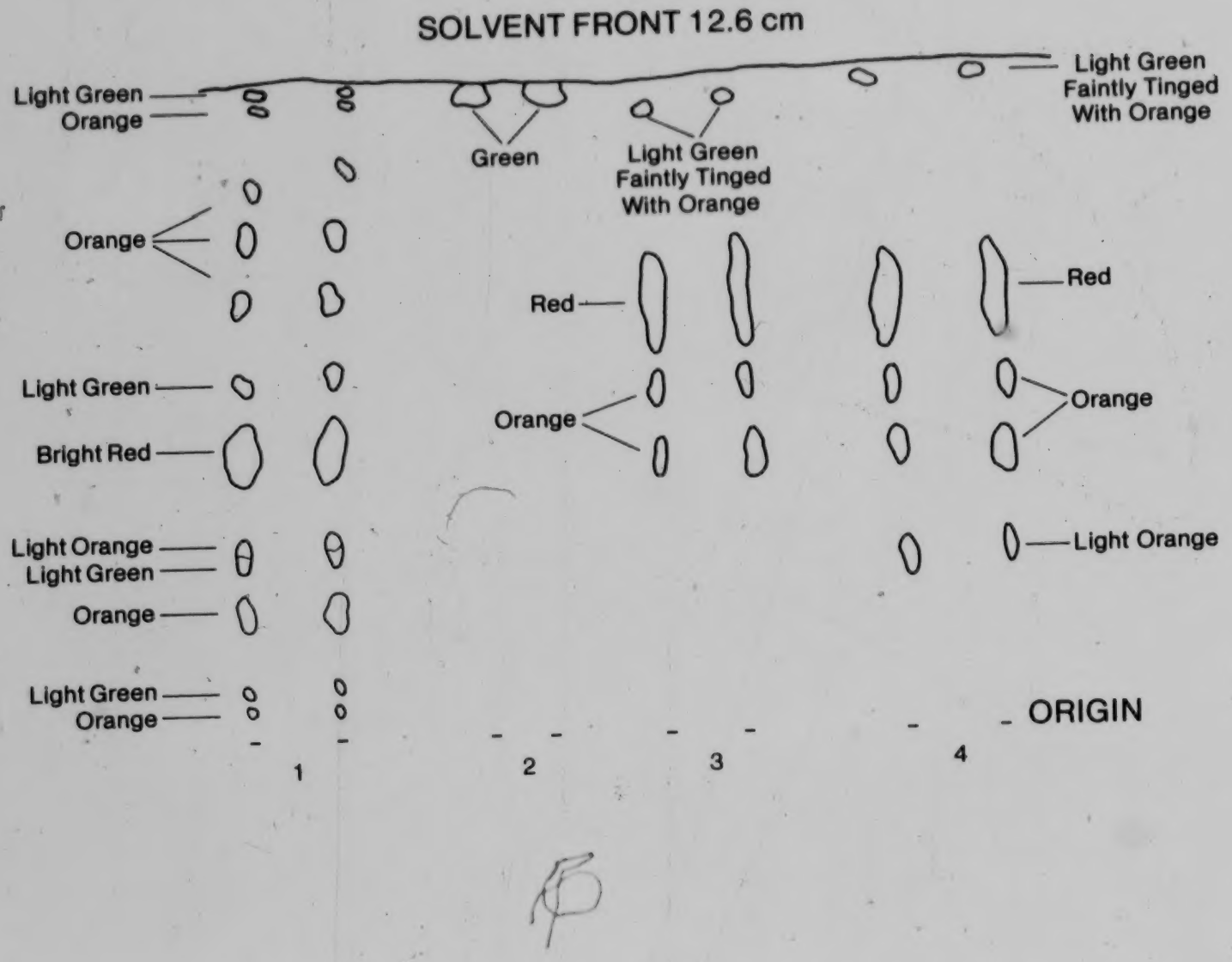
[c] The total number of surviving colonies on a complete medium containing a limiting concentration of adenine (MC with 5 µg adenine/ml).

[d] The abbreviations RP, RPW, R, RW, P, and PW refer to red-pink, red-pink-white, red, red-white, pink, and pink-white colonies, respectively.

[e] Represented by RP (twin-spot) and RPW colonies.

[f] Includes RP, RPW, R, RW, P, and PW colonies.

FIGURE 3. Diagram of a thin-layer chromatogram (Eastman Chromagram Sheet) developed in chloroform:ethanol (7:3) when viewed under long-wave (354-nm) UV light. The numbers 1, 2, 3, and 4 represent the light-exposed sample of medical grade I, monopotassium pamoate, medical grade I, and the USP reference standard of pyrvinium pamoate, respectively.


SOLVENT FRONT 12.6 cm
Light Green
Orange
Green
Light Green Faintly Tinged With Orange
Light Green Faintly Tinged With Orange
Orange
Red
Red
Light Green
Bright Red
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Light Orange
Light Green
Light Orange
Orange
Light Green
Orange
ORIGIN
1
2
3
4

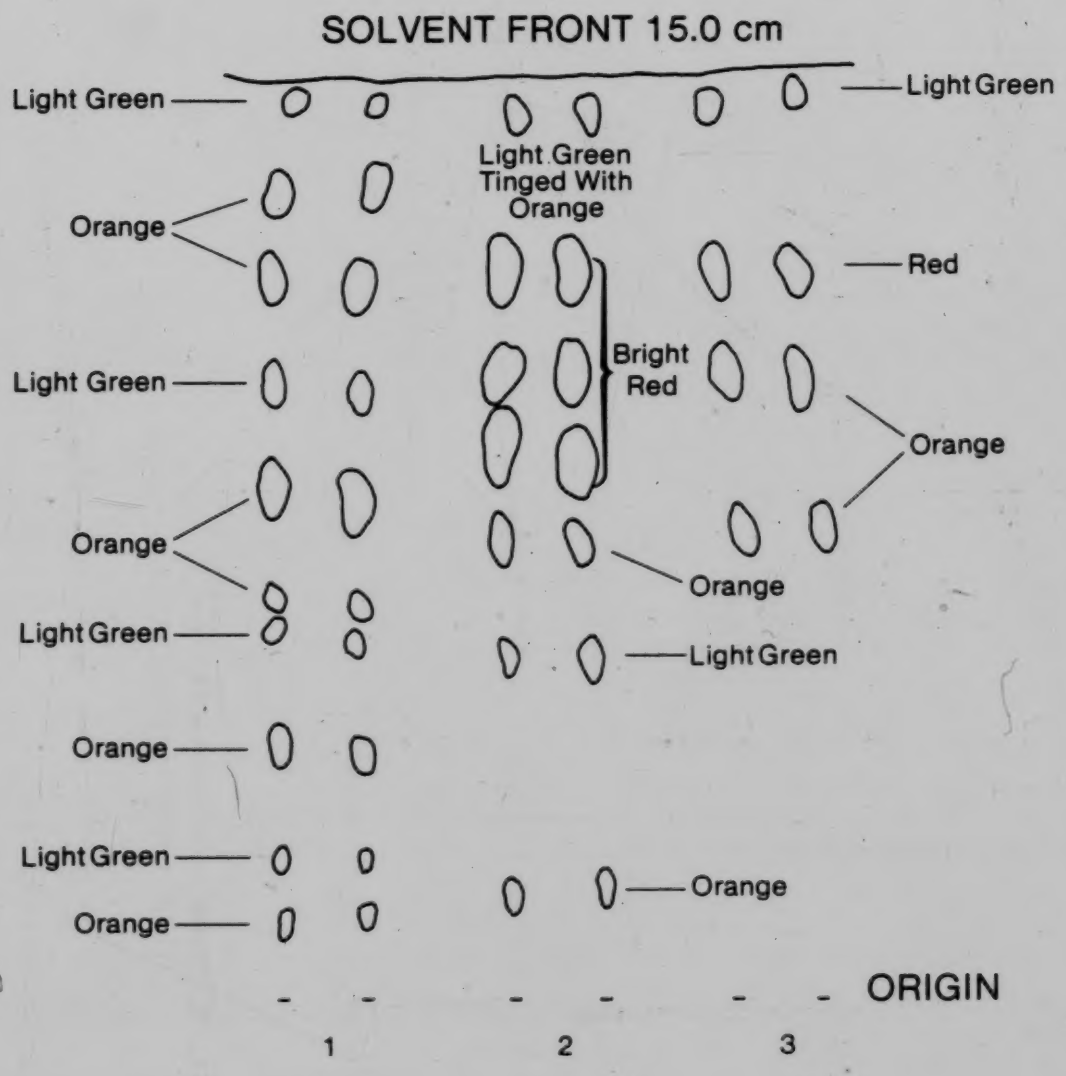


FIGURE 4. Diagram of a thin-layer chromatogram (Eastman Chromagram Sheet) developed in chloroform:ethanol (7:3) when viewed under long-wave (354-nm) UV light. The numbers 1, 2, and 3 represent the light-exposed sample of medical grade I, medical grade II, and medical grade I, respectively, of pyrvinium pamoate.

ARTICLE II

The Detection of Chemical Impurities by High Pressure Liquid Chromatography and the Genetic Activity of Medical Grades of Pyrvinium Pamoate in *Saccharomyces cerevisiae* and *Salmonella typhimurium*

ABSTRACT

The genetic activity of several medical grades of the anthelmintic drug pyrvinium pamoate was studied in a diploid mitotic recombination and gene conversion assay (strain D5 of *Saccharomyces cerevisiae*), and in several haploid yeast reversion assays (strains XV185-14C, XY718-1A, and 7854-1A of *Saccharomyces cerevisiae*). All of the samples were recombinogenic in strain D5 and mutagenic in the haploid strains, however, the degree of genetic activity varied considerably among the medical grades of pyrvinium pamoate that were tested. Similarly, these samples also varied in degree of mutagenicity when they were tested in strains TA98 and TA100 of *Salmonella typhimurium*, but some of the medical grades of pyrvinium pamoate were mutagenic both in the presence and in the absence of the metabolic transformation system, whereas other grades of the drug required such activation to be mutagenic. In addition, the medical grades and dosage forms of several brands of pyrvinium pamoate were examined for purity by fluorescence high pressure liquid chromatography (HPLC) using a methanol:water (90:10) solvent system. The tablets were not extracted quantitatively because the major purpose of the HPLC analyses was to determine the number of impurities, and their relative quantities, present in these pharmaceuticals. In general, there is a correlation between the degree of genetic activity and toxicity, and the number and relative quantity of impurities found in each sample.

INTRODUCTION

Pyrvinium pamoate (Figure 2) is an anthelmintic drug that is used in the treatment of pinworm infestations but the drug is not absorbed systemically (Rollo, 1980). Two doses of the drug (each dose at 7.5 mg/kg body weight) are administered orally in a two-week interval to eradicate the adult worm, *Enterobius (Oxyuris) vermicularis*. The increase in parasitic diseases (The Medical Letter, 1982) such as pinworm infestations and reinfestations, particularly among school children, has resulted in extensive prophylactic use of pyrvinium pamoate. This type of therapy is ineffective as pyrvinium pamoate is not active against the ova, nor is it active against adult pinworms when low doses of the drug are used. In Canada, several brands of pyrvinium pamoate are available to the general public as over-the-counter medications, whereas in the United States a prescription is required for this drug. Mexico does not have an official pharmacopeia and most drugs are readily accessible for self-medication.

Pyrvinium pamoate has been shown to be mutagenic in the *Salmonella typhimurium* reversion assay, and the S9 liver microsomal fraction was required to induce genetic activity in this test system (MacPhee and Podger, 1977; Lake and de la Iglesia, 1981); however, the medical grades of pyrvinium pamoate that are marketed in Mexico are also mutagenic in the absence of the metabolic activation system (Espinosa *et al.*, 1981; Cortinas de Nava *et al.*, 1983). The drug was also mutagenic in a haploid (strain XV185-14C) yeast reversion test (Galindo, 1979; Galindo *et al.*, 1979; Hennig *et al.*, 1981; Mehta *et al.*, 1982), and in a diploid (strain D5) mitotic recombination and gene conversion assay

(Hennig *et al.*, 1981; Mehta *et al.*, 1982). Pamoic acid was nonmutagenic in strains TA98 and TA100 of *Salmonella typhimurium* (Lake and de la Iglesia, 1981), and monopotassium pamoate was found to be nontoxic and nonmutagenic in strains XV185-14C and D5 of *Saccharomyces cerevisiae* (Mehta *et al.*, 1982). The monopyrvinium salts, pyrvinium chloride and pyrvinium iodide, are strong mutagens in both the haploid and diploid yeast assays (Mehta *et al.*, 1982), thus it is evident that the pyrvinium moiety is responsible for the genetic activity of pyrvinium pamoate.

Several medical grades of pyrvinium pamoate, which fulfilled the specifications described in the "United States Pharmacopeia" (USP XX, 1980), and the USP reference standard of the drug were examined for genetic activity and chemical purity.

METHODS AND MATERIALS

High Pressure Liquid Chromatography

Reversed-phase HPLC was performed utilizing a high pressure liquid chromatograph (Waters ALC 202/401) that was equipped with a μBondapak C_{18} column. This reversed-phase column consists of a monomolecular layer of organosilane that is bonded to a pellicular silica packing. The chromatographic solvent system was methanol:water (90:10), the solvent flow rate was 0.5 ml/min at an inlet pressure of 2500 psig, and the chart speed was set at 0.5 cm/min.

The fluorescent intensity of the column eluent was measured continuously with a fluorometer that was equipped with an entrance filter (range 300-480 nm) and a 389-nm emission filter (range 375-405 nm).

The excitation wavelength was set at 355 nm and the range and sensitivity were maintained at 1.0 μA and 4.00, respectively.

Pyrvinium Pamoate

Monopotassium pamoate (Sigma Chemical Company), pyrvinium iodide (Delmar Chemicals), the USP reference standard (A) of pyrvinium pamoate (lot F2), and medical grades B, C, D, and E of pyrvinium pamoate were used as supplied by the manufacturers and prepared for HPLC analysis by dissolution in methoxyethanol to a final concentration of approximately 1 mg/ml. The medical grades and dosage forms of pyrvinium pamoate were supplied by Parke-Davis Canada Incorporated, Charles E. Frosst & Company, ICN Canada Limited, and Laboratorios Columbia.

For the mutagenicity assays, 16 mM stock solutions (18.4 mg/ml) of the USP reference standard and each medical grade of pyrvinium pamoate were prepared in dimethylsulfoxide (DMSO); when required, the stock solutions were diluted with DMSO. The solutions were stored in glassware that was wrapped with aluminum foil to prevent the light-catalyzed degradation of pyrvinium pamoate (Beckstead and Smith, 1967; Mehta *et al.*, 1982).

Dosage Forms

One tablet of each of three brands of pyrvinium pamoate (B, C, and D) was finely powdered with the aid of a mortar and pestle, dissolved in 15 ml of methanol, agitated manually for 5 minutes, and then allowed to settle. The tablet excipients precipitated on standing and the supernatant was used in the HPLC analysis.

The tablets were not assayed for drug content and the labeled strength of each tablet (75 mg pyrvinium pamoate) was assumed. In the

qualitative HPLC analyses, the concentration of pyrvinium pamoate in the methanol supernatant was estimated to be 5 mg/ml.

Yeast Strains

Strain XV185-14C of *Saccharomyces cerevisiae* has the genotype

a *ade2-1 arg4-17 lys1-1 trp5-48 his1-7 hom3-10*;

his1-7 is a missense mutation, *hom3-10* is a putative frameshift mutation, and *ade2-1*, *arg4-17*, *lys1-1*, and *trp5-48* are ocher nonsense mutations. Strains XY718-1A (genotype a *his4-519 leu2-3*) and 7854-1A (genotype a *his4-38 leu2-3*) contain the frameshift mutations *his4-519*, *his4-38*, and *leu2-3* (Culbertson *et al.*, 1977).

The diploid strain D5 of *Saccharomyces cerevisiae* (Zimmermann, 1973) is heteroallelic for two complementing mutations of the *ade2* locus: *ade2-119*, which is a slightly leaky allele with a pink phenotype, and *ade2-40*, which is a nonleaky allele with a red phenotype. The genotype of this strain is $\frac{\alpha}{a}\ \frac{trp1\text{-}1}{+}\ \frac{ade2\text{-}40}{ade2\text{-}119}\ \frac{MAL1}{+}\ \frac{+}{MAL4}$.

Bacterial Strains

The *Salmonella typhimurium* strains TA98 (genotype *hisD3052 rfa ΔuvrB*) and TA100 (genotype *hisG46 rfa ΔuvrB*), both of which carry the pKM101 plasmid, were donated by Dr. Bruce N. Ames (University of California at Berkeley). The strains were stored at -70°C and their genetic markers have been checked routinely for histidine auxotrophy, sensitivity to crystal violet, sensitivity to ultraviolet radiation, and ampicillin resistance, as described by Ames *et al.* (1975), and by Maron and Ames (1983).

Mutagenicity Assays

Yeast assays. Stationary-phase cells of strains D5, XV185-14C, XY718-1A, and 7854-1A were suspended in phosphate buffer ($2x10^8$ cells/ml), as described by Mehta *et al.* (1982). Either 0.1 ml of a stock solution of pyrvinium pamoate, 0.1 ml of DMSO, or 0.1 ml of double-distilled water was added to 3.9 ml of the cell suspension of each yeast strain. The reaction mixtures were incubated at 30°C in a water-bath shaker and aliquots were withdrawn at 2-, 3-, 6-, 10-, and 24-hour intervals. These samples were washed twice and a series of dilutions, wherever necessary, were prepared in phosphate buffer (pH 7.0).

The various media that were used have been described previously (von Borstel *et al.*, 1971). Mortimer complete (MC) medium with a growth-limiting concentration of adenine (5 µg/ml) was used to score for mitotic recombination and gene conversion in strain D5, whereas omission media were used to detect revertants in strains XV185-14C, XY718-1A, and 7854-1A. The plates were incubated at 30°C and scored after 5-10 days. Coreversion analyses were carried out with strains XY718-1A and 7854-1A by replica-plating the *HIS*$^+$ and *LEU*$^+$ revertants onto MC minus leucine and MC minus histidine media, respectively.

Bacterial assays. Strains TA98 and TA100 of *Salmonella typhimurium* were cultured in L-broth (tryptone 1.2%, NaCl 1.0%, yeast extract 0.5%, dextrose 0.1%) at 37°C for 16 hours to attain stationary-phase cells. The mutagenicity assays were performed as described by Ames *et al.* (1975), and by Maron and Ames (1983). Each treatment tube contained 0.1 ml of stationary-phase bacterial culture (approximately 10^8 bacteria), 0.1 ml of drug solution (dissolved in DMSO) or DMSO, 0.5 ml S9 mix or 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4), 2.0 ml of

molten top agar (0.6% Difco agar and 0.5% NaCl), and 0.2 ml of 0.5 mM histidine·HCl-0.5 mM biotin solution.

The contents of the treatment tubes were poured onto minimal-glucose agar plates (Vogel-Bonner E medium plus 1.5% Difco agar and 2.0% glucose). Once the top agar had solidified, the plates were incubated at 37°C and, after 48 hours of total incubation time, the *his*$^+$ revertants were scored.

Each ml of S9 mix contained 0.1 ml rat liver homogenate, 3.0 µmoles NADP, 3.0 µmoles glucose-6-phosphate, 8.0 µmoles $MgCl_2$, 33.0 µmoles KCl, and 84.7 µmoles of 0.1 M sodium phosphate buffer (pH 7.4). The activity of the S9 liver microsomal fraction was confirmed with positive controls that required metabolic activation to be mutagenic; cyclophosphamide (Sigma Chemical Company) induces base-pair substitutions and 2-acetylaminofluorene (Lancaster Synthesis Limited) causes frameshift mutations.

RESULTS

The difference in the physical appearance of the various medical grades of pyrvinium pamoate was apparent immediately. The USP reference standard (A) and medical grades B and C were bright red in color, whereas the color of products D and E was a dark brown. The mono-pyrvinium salts, pyrvinium chloride and pyrvinium iodide, were also dark brown in color. Similarly, a sample of medical grade B that had been exposed to room and window light for 9 months was dark brown in color. These color differences also appeared in the methanol extracts

of the tablets, except that the extract of tablet C was a much darker red color than that of tablet B and, in addition, it had acquired a brownish hue.

HPLC Analysis

Although normal-phase thin-layer chromatography (TLC) using a chloroform:ethanol (7:3) solvent system provided an adequate separation of the components of pyrvinium pamoate (Beckstead and Smith, 1967; Mehta *et al.*, 1982), TLC was not a suitable separation technique for a compound as unstable as pyrvinium pamoate because the preparative TLC procedure itself appeared to promote the rapid breakdown of the various components. The high affinity of pyrvinium pamoate for the silica gel rendered a quantitative extraction difficult to achieve (H.D. Beckstead and C.H. Barnstein, personal communications; Mehta *et al.*, 1982) and excluded the use of normal-phase HPLC. Reversed-phase HPLC has been found to be the most suitable method for separating the impurities in medical grades of pyrvinium pamoate.

The optimum conditions for the HPLC analysis of pyrvinium pamoate include a solvent system that contains at least 80% of a polar organic solvent such as methanol, and a solvent flow rate between 0.5 ml and 1.0 ml/min. If less than 80% of organic solvent was used, the column required frequent rinsing with 100% methanol and lengthy re-equilibration periods with the eluting solvent system.

The separation of the pamoate moiety from the pyrvinium moiety was achieved readily with a methanol:water (90:10) solvent system. The identities of the pamoate and pyrvinium moieties were confirmed with the fluorescence high pressure liquid chromatograms of the appropriate

reference standards, which were monopotassium pamoate and pyrvinium iodide (Figure 5). The *trans*-isomer of pyrvinium iodide, which appears to be the most abundant of the geometric isomers (this volume, p. 129), had a retention time of 8 minutes (Figure 5).

In the separation of the components of pyrvinium pamoate, the pamoate fraction had a shorter retention time (4 minutes) than did the pyrvinium moiety (8 minutes), and it was always the first component that was eluted from the column (Figures 6 and 7). The additional peak (at a retention time of 10 minutes) in the USP reference standard of pyrvinium pamoate (Figure 6, A) appears to correspond to the additional spot at R_f 0.26 (see Table 2 and Figure 3) that was observed when the USP reference standard and a medical grade of the drug were compared by TLC analysis (Beckstead and Smith, 1967; Mehta *et al.*, 1982). An examination of the HPLC data (Figure 6) reveals that medical grades C, D, and E contain greater quantities of the pyrvinium moiety than do samples A and B. This is apparent when the peak height is compared to the quantity of pyrvinium pamoate that had been injected.

Medical grades B and C of pyrvinium pamoate (Figure 6) and the methanol extracts of their respective dosage forms (Figure 7) did not differ significantly in chromatographic character. It is evident in Figures 6 and 7 that medical grades C and D and their tablets, as well as medical grade E, contained a greater number of separable components than did medical grade B and its tablets. The number and relative quantities of the impurities were much greater in tablet D than in the corresponding medical grade, and the component at a retention time of 10 minutes (Figure 7) was especially prominent. Medical grade B of pyrvinium pamoate and its tablets exhibited the fewest impurities.

Mutagenicity Studies

The USP reference standard (A) and medical grades B and C of pyrvinium pamoate did not exhibit genetic activity in the diploid strain D5 of *Saccharomyces cerevisiae* after a 2-hour treatment period, but after longer periods of exposure (6, 10, and 24 hours) these three samples of the drug induced mitotic recombination and increased the frequency of aberrant colonies (Table 9). In contrast, medical grades D and E of pyrvinium pamoate induced significant increases in mitotic recombination and aberrant phenotypes after both shorter and longer incubation periods. As is demonstrated by the survival data in Table 9, samples D and E were quite toxic to the cells, however, greatly enhanced levels of aberrant colonies and mitotic recombination were produced, even as the survival declined.

In the haploid strain XV185-14C of *Saccharomyces cerevisiae*, the USP reference standard (A) and medical grades B and C of pyrvinium pamoate did not increase the frequency of *HIS*$^+$ and *HOM*$^+$ revertants until the cells had been treated for 24 hours, whereas considerable increases in the frequency of *HIS*$^+$, *ARG*$^+$, and *HOM*$^+$ revertants were observed with medical grades D and E after 2 hours or more of treatment (Table 10). Medical grades D and E are potent mutagens and, as is indicated by the survival data in Table 10, both samples were highly toxic; the reversion frequencies continued to increase with prolonged incubation periods despite this drastic decrease in survival.

In strains XY718-1A and 7854-1A of *Saccharomyces cerevisiae*, the USP reference standard (A) and medical grades B and C of pyrvinium pamoate increased the frequencies of *HIS*$^+$ and *LEU*$^+$ revertants above the spontaneous levels (Tables 11 and 12). An enhancement of these

reversion frequencies was observed as the time of exposure to samples A, B, and C was increased. Strain 7854-1A appeared to be more sensitive to the toxic effects of pyrvinium pamoate since it had a lower survival rate than strain XY718-1A at 10 hours and 24 hours of exposure to the drug. The results of the coreversion tests that were performed on the *HIS*$^+$ revertants indicate that the frequencies of locus and suppressor events were not significantly different (Tables 11 and 12), however, there is a discernible trend toward reversion at the locus in strain 7854-1A, although this tendency is not entirely consistent (Table 12). The coreversion analyses of the *LEU*$^+$ revertants revealed that the majority of the reversion events had occurred at the *leu2-3* locus, with very few suppressor events (Tables 11 and 12). Medical grades D and E of pyrvinium pamoate increased the *HIS*$^+$ and *LEU*$^+$ reversion frequencies considerably and appeared to induce locus and suppressor events with equal frequencies (Tables 11 and 12). Due to the toxicity of medical grades D and E, no revertants were obtained at 24 hours of exposure. It is possible that lower concentrations of medical grades D and E would induce detectable levels of revertants in strains XY718-1A and 7854-1A at 24 hours.

A noteworthy observation is the apparent increase in viability of the cell samples that had been treated with medical grades A, B, or C of pyrvinium pamoate (Tables 9-12). This is an artefact that was due to the effect of a semisolid matrix, which formed in each of the treatment tubes during centrifugation because of the limited aqueous solubility of pyrvinium pamoate (Mehta *et al.*, 1982). The drug matrix trapped many of the cells, thus fewer cells were lost from the treatment tubes, as compared to the control tubes, in the subsequent washing procedures.

The USP reference standard (A) and medical grades B and C of pyrvinium pamoate did not increase the number of *his*$^+$ revertants above the spontaneous level in strains TA100 and TA98 of *Salmonella typhimurium* when the metabolic activation system was absent. In fact, these particular samples appeared to be toxic since a continuous bacterial lawn was not visible on the plates, and the number of revertants induced by DMSO, the negative control, was 3-fold higher in strain TA100 without the S9 fraction (Table 13). Metabolic activation was required to elicit an approximately 5-fold increase of revertants in strain TA100 and a 7-fold increase in strain TA98 when samples A, B, and C of pyrvinium pamoate are compared to DMSO (Table 13). In comparison to the small number of *his*$^+$ revertants that were induced by samples A, B, and C in the absence of mammalian microsomes, the addition of the S9 mix to the bacterial assay resulted in a 10-fold increase of revertants in strain TA100 and a 2-fold increase in strain TA98 (Table 13).

In strains TA100 and TA98 of *Salmonella typhimurium*, medical grades D and E of pyrvinium pamoate increased the number of *his*$^+$ revertants 2.5-fold over the control in the absence of rat liver microsomal enzymes, and 4-fold in the presence of the S9 mix (Table 13). The mutagenicity of samples D and E in strain TA100 was not enhanced by rat liver microsomes, and less than a 2-fold difference in the number of histidine prototrophs was evident in strain TA98 when the S9 mix was present (Table 13). Medical grades D and E of pyrvinium pamoate were highly mutagenic, both in the presence and in the absence of the metabolic transformation system.

DISCUSSION

The results of the experiments in *Saccharomyces cerevisiae* (strains D5, XV185-14C, XY718-1A, and 7854-1A) and *Salmonella typhimurium* (strains TA100 and TA98) have demonstrated that medical grades D and E of pyrvinium pamoate are potent mutagens in, and highly toxic to, yeast and bacteria. All of the samples of pyrvinium pamoate induced dramatic increases in the frequency of mitotic recombination, when compared with the frequencies of other types of aberrant colonies, in strain D5 of *Saccharomyces cerevisiae*. Mitotic recombinants represent approximately 50% of the total aberrant phenotypes after 6 hours and 10 hours of exposure, and about 75% of the genetic events after 10 hours and 24 hours of exposure (Table 9).

The USP reference standard (A) and medical grades B and C of pyrvinium pamoate required a long treatment period (24 hours) to elicit a mutagenic response in strain XV185-14C of *Saccharomyces cerevisiae*, whereas medical grades D and E were powerfully mutagenic at only 2 hours of exposure (Table 10). All of the samples of pyrvinium pamoate were mutagenic in strain XV185-14C, inducing base-pair substitution mutations at the *his1-7* locus and frameshift mutations at the *hom3-10* locus. *ARG*$^+$ revertants are believed to arise primarily from the induction of base-pair substitutions of the transversion class (von Borstel *et al.*, 1973). As is evident in Table 10, whenever mutation induction takes place the frequency of *ARG*$^+$ revertants is quite low when compared with the frequency of *HIS*$^+$ revertants. Since reversion to *HIS*$^+$ can occur through base-pair transitions, it seems likely that most of the mutations that are induced by medical grades A, B, and C of pyrvinium

pamoate are base-pair substitutions of the transition class.

All of the medical grades of pyrvinium pamoate induced frameshift mutations in strains XY718-1A and 7854-1A of *Saccharomyces cerevisiae*. The results of the coreversion tests performed on the *HIS*$^+$ revertants indicate that pyrvinium pamoate causes locus and suppressor events with equal frequencies, whereas the coreversion analyses of the *LEU*$^+$ revertants revealed predominantly *leu2* locus events.

In *Salmonella typhimurium*, the USP reference standard (A) and medical grades B and C of pyrvinium pamoate induced base-pair substitution mutations (detected with strain TA100) and frameshift mutations (detected with strain TA98) only in the presence of the rat-liver metabolic transformation system. Therefore, pyrvinium pamoate, as characterized by samples A, B, and C, is a promutagen that is converted to a mutagen by mammalian liver microsomes.

In the presence of the S9 mix, medical grades D and E were 44 times as mutagenic as samples A, B, and C in strain TA100, and 32 times as mutagenic in strain TA98, when their specific potencies (revertants/μg medical grade) were compared (Table 13). Medical grades D and E of pyrvinium pamoate induced frameshift and base-pair substitution mutations in *Salmonella typhimurium*, both with and without metabolic activation. Clearly, these medical grades of pyrvinium pamoate contain a contaminant (or contaminants) that does not require metabolic activation to be mutagenic. All of the samples of this drug exhibited genetic activity in the yeast assays because yeast has polysubstrate monooxygenases (cytochrome P-450), which can metabolize promutagens into mutagens (Callen and Philpot, 1977).

In general, the apparent correlation between the degree of genetic

activity and toxicity of the medical grades of pyrvinium pamoate, and the number and relative quantity of impurities found in each sample, is particularly striking. Although these impurities may account for the enhanced genetic activity of medical grades D and E (Tables 9-13), there is compelling evidence that suggests that the pyrvinium concentration is the major contributing factor. First, it has already been established that light-catalyzed degradation, hence the improper storage of the drug, is not responsible for the genetic activity of pyrvinium pamoate (Mehta *et al.*, 1982). Second, the highly mutagenic sample of pyrvinium pamoate, which is comparable to medical grades D and E in genetic activity, was nearly as active genetically as the monopyrvinium salts, pyrvinium chloride and pyrvinium iodide, in strain D5 of yeast (Mehta *et al.*, 1982). Third, the discrepancy between the peak height and the relative quantity of pyrvinium pamoate that had been injected is particularly striking. For example, 10 µg of B and 5.5 µg of D were analyzed under identical experimental conditions (Figure 6), yet D appears to contain twice as much pyrvinium when compared to B. It seems obvious that this discrepancy is due to the presence of monopyrvinium salts in some of the medical grades of pyrvinium pamoate.

A published method for the synthesis of pyrvinium pamoate involves the reaction between pyrvinium methylsulfate and disodium pamoate (Swinyard and Harvey, 1970; Swinyard, 1980). An incomplete chemical reaction between a monopyrvinium salt and a pamoate salt during the synthetic procedure could be expected, and this would account for the presence of monopyrvinium salts in certain medical grades of pyrvinium pamoate.

Medical grade C is not more mutagenic than samples A and B (Tables

9-13), yet it is obvious that medical grade C has a higher pyrvinium content (Figure 6), thus it would be expected to exhibit enhanced mutagenicity, although not to the levels of medical grades D and E. Until more information is available on the identity and purity of the reagents and precursors that are used in the synthesis of pyrvinium pamoate, the question of the low genetic activity of medical grade C must remain unresolved.

Monopyrvinium salts, such as pyrvinium chloride and pyrvinium iodide, are absorbed systemically and cause renal damage that was believed to be reversible upon the withdrawal of the drug (Hales and Welch, 1953; Weston *et al.*, 1953). Pyrvinium pamoate is not absorbed significantly from the gastrointestinal tract (Rollo, 1980) and it is less active genetically than either pyrvinium chloride or pyrvinium iodide (Mehta *et al.*, 1982). Monopyrvinium salts may be present as contaminants or incomplete reactants in some medical grades of pyrvinium pamoate and this could account for the enhanced genetic activity of medical grades D and E (Tables 9-13).

TABLE 9

Induction of mitotic recombination and aberrant colonies in strain D5 of *Saccharomyces cerevisiae* after treatment with several medical grades of pyrvinium pamoate (4×10^{-4} M)

Medical Grade	Exposure Time (hours)	Survival (%)	Total Colonies[a]	Colony Phenotype[b]						Mitotic Recombination[c] (%)	Total Aberrant Colonies[d] (%)
				RP	RPW	R	RW	P	PW		
control (2.5% DMSO)	2	100	1002	0	1	1	0	0	1	0.1	0.3
	6	100	1038	0	0	0	0	0	0	<0.1	<0.1
	10	100	990	0	0	1	0	0	0	<0.1	0.1
	24	100	1094	0	0	1	3	2	0	<0.1	0.6
A[e]	2	112	1117	2	0	0	0	0	1	0.2	0.3
	6	96	1000	4	2	0	0	0	5	0.6	1.1
	10	95	936	5	1	0	1	5	6	0.9	1.9
	24	94	1025	9	3	2	2	2	4	1.2	2.2
B	2	114	1137	2	0	0	0	2	1	0.2	0.4
	6	95	983	5	0	0	0	0	2	0.5	0.7
	10	95	937	12	3	0	0	1	3	1.6	2.0
	24	94	1027	12	3	1	3	1	1	1.5	2.0
C	2	112	1124	2	0	0	0	1	1	0.2	0.4
	6	97	1010	6	1	0	1	4	1	0.7	1.3
	10	96	946	11	2	0	0	0	6	1.4	2.0
	24	94	1030	9	3	3	1	3	3	1.2	2.1

TABLE 9 (continued)

Medical Grade	Exposure Time (hours)	Survival (%)	Total Colonies[a]	Colony Phenotype[b]						Mitotic Recombination[c] (%)	Total Aberrant Colonies[d] (%)
				RP	RPW	R	RW	P	PW		
D	2	98	3131	23	6	9	1	3	24	0.93	2.1
	6	69	1922	30	8	7	3	10	15	2.0	3.8
	10	33	882	13	5	8	1	16	5	2.0	5.4
	24	16	402	9	0	0	1	1	2	2	3.2
E	2	89	2863	28	7	3	5	4	12	1.2	2.1
	6	65	1803	29	9	4	0	14	17	2.1	4.0
	10	51	1336	21	6	6	0	13	8	2.0	4.0
	24	34	854	18	4	2	2	6	2	2.6	4.0

[a]The total number of surviving colonies on a complete medium (YEPD-agar) that contains a low concentration of adenine.

[b]The abbreviations RP, RPW, R, RW, P, and PW refer to red-pink, red-pink-white, red, red-white, pink, and pink-white colonies, respectively.

[c]Represented by RP (twin-spot) and RPW colonies.

[d]Includes RP, RPW, R, RW, P, and PW colonies.

[e]The USP reference standard of pyrvinium pamoate (lot F2).

TABLE 10

Reversion frequencies induced in strain XV185-14C of *Saccharomyces cerevisiae* after treatment with several medical grades of pyrvinium pamoate (4×10^{-4} M)

Medical Grade	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)		
			HIS$^+$	*ARG*$^+$	*HOM*$^+$
control (2.5% DMSO)	2	100 (475)	14 (26)	7 (14)	3 (13)
	6	100 (468)	17 (32)	7 (13)	3 (11)
	10	100 (469)	18 (34)	11 (20)	3 (11)
	24	100 (476)	20 (39)	14 (27)	1 (4)
A[c]	2	109 (519)	14 (28)	18 (37)	3 (14)
	6	101 (471)	18 (33)	8 (15)	4 (14)
	10	100 (470)	20 (37)	11 (20)	4 (16)
	24	100 (474)	90 (170)	18 (34)	40 (150)
B	2	111 (527)	19 (40)	19 (40)	4 (16)
	6	104 (488)	22 (44)	12 (24)	4 (14)
	10	101 (474)	28 (54)	13 (25)	5 (18)
	24	100 (475)	100 (190)	20 (38)	42 (138)
C	2	110 (520)	14 (29)	18 (38)	3 (14)
	6	103 (484)	22 (42)	11 (22)	3 (12)
	10	101 (472)	22 (42)	13 (24)	4 (17)
	24	99 (472)	86 (162)	15 (29)	30 (112)

TABLE 10 (continued)

Medical Grade	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)		
			HIS$^+$	*ARG*$^+$	*HOM*$^+$
D	2	62 (1176)	77 (90)	18 (21)	11 (25)
	6	44 (818)	202 (165)	32 (26)	23 (33)
	10	35 (658)	471 (310)	97 (64)	45 (59)
	24	3 (58)	1000 (58)	100 (7)	80 (7)
E	2	66 (1251)	64 (80)	14 (17)	11 (27)
	6	48 (903)	234 (211)	44 (40)	18 (29)
	10	39 (724)	501 (363)	80 (58)	41 (60)
	24	4 (68)	1910 (130)	60 (4)	130 (16)

[a]The numbers in parentheses constitute the total number of revertant colonies, as determined by plate counts.

[b]The numbers in parentheses constitute the total number of surviving colonies on 4 plates of YEPD-agar.

[c]The USP reference standard of pyrvinium pamoate (lot F2).

TABLE 11

Reversion frequencies induced in strain XY718-1A (a *his4-519 leu2-3*) of *Saccharomyces cerevisiae* after treatment with several medical grades of pyrvinium pamoate (4×10^{-4} M)

Medical Grade	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)					
			HIS$^+$			*LEU*$^+$		
			Locus	Suppressors	Total	Locus	Suppressors	Total
control (2.5% water)	3	100 (965)	<0.3 (0)	<0.3 (0)	<0.3 (0)	<0.3 (0)	<0.3 (0)	<0.3 (0)
	10	100 (987)	<0.3 (0)	<0.3 (0)	<0.3 (0)	0.5 (2)	<0.3 (0)	0.5 (2)
	24	100 (933)	<0.3 (0)	<0.3 (0)	<0.3 (0)	0.8 (3)	<0.3 (0)	0.8 (3)
control (2.5% DMSO)	3	100 (964)	<0.3 (0)	<0.3 (0)	<0.3 (0)	0.5 (2)	<0.3 (0)	0.5 (2)
	10	99 (980)	<0.3 (0)	<0.3 (0)	<0.3 (0)	<0.3 (0)	0.5 (2)	0.5 (2)
	24	99 (925)	<0.3 (0)	0.3 (1)	0.3 (1)	0.3 (1)	0.5 (2)	0.8 (3)
A[c]	3	102 (983)	0.2 (1)	<0.2 (0)	0.2 (1)	0.5 (2)	<0.2 (0)	0.5 (2)
	10	97 (958)	<0.3 (0)	0.3 (1)	0.3 (1)	0.8 (3)	<0.3 (0)	0.8 (3)
	24	96 (897)	0.6 (2)	0.3 (1)	0.8 (3)	0.8 (3)	0.8 (3)	2 (6)
B	3	103 (998)	<0.2 (0)	0.5 (2)	0.5 (2)	2 (7)	<0.2 (0)	2 (7)
	10	98 (962)	0.5 (2)	<0.3 (0)	0.5 (2)	2 (6)	0.3 (1)	2 (7)
	24	97 (905)	0.3 (1)	0.8 (3)	1 (4)	2 (7)	<0.3 (0)	2 (7)
C	3	101 (974)	<0.3 (0)	<0.3 (0)	<0.3 (0)	0.5 (2)	0.8 (3)	1 (5)
	10	98 (963)	0.3 (1)	<0.3 (0)	0.3 (1)	2 (8)	0.3 (1)	2 (9)
	24	97 (901)	<0.3 (0)	0.6 (2)	0.6 (2)	2.8(10)	<0.3 (0)	2.8(10)

TABLE 11 (continued)

Medical Grade	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)											
			HIS$^+$						*LEU*$^+$					
			Locus		Suppressors		Total		Locus		Suppressors		Total	
D	3	5 (204)	10	(3)	5	(1)	20	(4)	5	(1)	5	(1)	10	(2)
	10	5 (199)	20	(3)	10	(2)	30	(5)	20	(4)	5	(1)	30	(5)
	24	2 (80)	<10	(0)	<10	(0)	<10	(0)	<10	(0)	<10	(0)	<10	(0)
E	3	17 (659)	3	(2)	<2	(0)	3	(2)	2	(1)	5	(3)	6	(4)
	10	13 (522)	4	(2)	4	(2)	8	(4)	10	(5)	6	(3)	20	(8)
	24	6 (234)	<4	(0)	<4	(0)	<4	(0)	<4	(0)	<4	(0)	<4	(0)

[a]The numbers in parentheses constitute the total number of revertant colonies, as determined by plate counts. Suppressor events were identified by corevertants (His$^+$Leu$^+$ phenotype). Wherever revertant counts of zero appear, reversion frequencies of less than the value corresponding to one revertant colony are reported.

[b]The numbers in parentheses constitute the total number of surviving colonies on 4 plates of YEPD-agar.

[c]The USP reference standard of pyrvinium pamoate (lot F2).

TABLE 12

Reversion frequencies induced in strain 7854-1A (a *his4-38 leu2-3*) of *Saccharomyces cerevisiae* after treatment with several medical grades of pyrvinium pamoate (4×10^{-4} M)

Medical Grade	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)					
			HIS$^+$			*LEU*$^+$		
			Locus	Suppressors	Total	Locus	Suppressors	Total
control (2.5% water)	3	100 (625)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)
	10	100 (725)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)
	24	100 (706)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)
control (2.5% DMSO)	3	99 (621)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)
	10	98 (709)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)
	24	92 (647)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	<0.4 (0)	0.4 (1)
A[c]	3	103 (646)	0.4 (1)	0.4 (1)	0.8 (2)	0.4 (1)	<0.4 (0)	0.4 (1)
	10	84 (610)	0.8 (2)	0.8 (2)	2 (4)	1 (3)	<0.4 (0)	1 (3)
	24	83 (585)	0.4 (1)	1 (3)	2 (4)	0.8 (2)	0.4 (1)	1 (3)
B	3	107 (670)	1 (4)	0.8 (2)	2 (6)	0.8 (2)	<0.4 (0)	0.8 (2)
	10	85 (614)	2 (5)	0.8 (2)	3 (7)	0.8 (2)	0.4 (1)	1 (3)
	24	84 (592)	2 (5)	1 (3)	3 (8)	2 (4)	<0.4 (0)	2 (4)
C	3	105 (657)	0.8 (2)	0.8 (2)	2 (4)	0.8 (2)	<0.4 (0)	0.8 (2)
	10	86 (625)	2 (5)	1 (3)	3 (8)	2 (4)	<0.4 (0)	2 (4)
	24	84 (595)	4 (9)	0.4 (1)	4.2(10)	3 (6)	<0.4 (0)	3 (6)

TABLE 12 (continued)

Medical Grade	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)					
			HIS+			*LEU*+		
			Locus	Suppressors	Total	Locus	Suppressors	Total
D	3	5 (29)	20 (2)	9 (1)	30 (3)	<9 (0)	20 (2)	20 (2)
	10	1 (10)	20 (1)	<20 (0)	20 (1)	20 (1)	<20 (0)	20 (1)
	24	1 (8)	<30 (0)	<30 (0)	<30 (0)	<30 (0)	<30 (0)	<30 (0)
E	3	10 (65)	20 (4)	20 (4)	30 (8)	4 (1)	<4 (0)	4 (1)
	10	5 (37)	30 (5)	<7 (0)	30 (5)	<7 (0)	<7 (0)	<7 (0)
	24	4 (29)	<9 (0)	<9 (0)	<9 (0)	<9 (0)	<9 (0)	<9 (0)

[a]The numbers in parentheses constitute the total number of revertant colonies, as determined by plate counts. Suppressor events were identified by corevertants (His^+Leu^+ phenotype). Wherever revertant counts of zero appear, reversion frequencies of less than the value corresponding to one revertant colony are reported.

[b]The numbers in parentheses constitute the total number of surviving colonies on 4 plates of YEPD-agar.

[c]The USP reference standard of pyrvinium pamoate (lot F2).

TABLE 13

Mutagenicity of several medical grades of pyrvinium pamoate in strains TA100 and TA98 of *Salmonella typhimurium*

Medical Grade	Concentration (µg/petri plate)	Revertants/plate[a]				Revertants/µg			
		TA100		TA98		TA100		TA98	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
control (100 µl DMSO)	---	109±10	74±37	78±11	34±18	--	--	--	--
A[b]	250	27±11	360±47	94±63	233±21	0.1	1.4	0.4	0.9
B	250	54±45	354±82	108±46	225±19	0.2	1.4	0.4	0.9
C	250	24±12	316±70	98±24	233±21	0.1	1.3	0.4	0.9
D	5	330±61	306±76	205±57	141±17	66	61	41	28
E	5	226±69	293±46	203±36	155±41	45	59	41	31

[a]Average of 4 replicate plates ± the standard deviation; -S9 and +S9 denote the absence and presence of the rat liver homogenate.

Values obtained with the positive controls: cyclophosphamide (200 µg/plate), 129±22 (-S9) and 262±36 (+S9) in TA100; 2-acetylaminofluorene (10 µg/plate), 73±13 (-S9) and 1045±31 (+S9) in TA98.

[b]The USP reference standard of pyrvinium pamoate (lot F2).

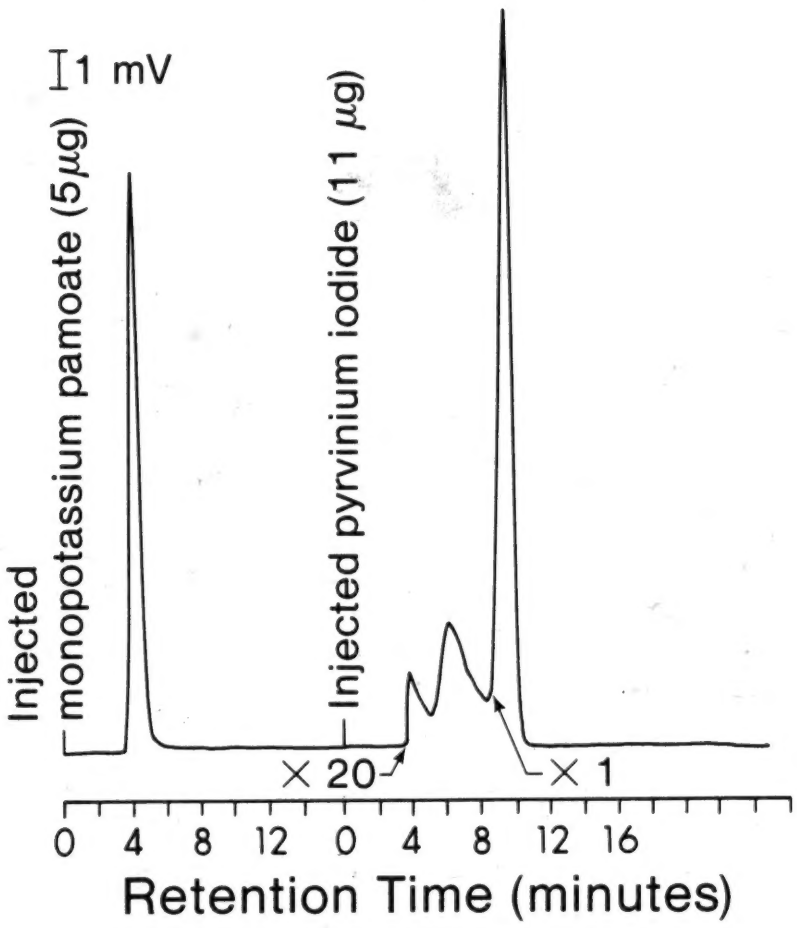


FIGURE 5. Fluorescence high pressure liquid chromatograms for monopotassium pamoate (injection volume 5 μl) and pyrvinium iodide (injection volume 10 μl), respectively. The methanol:water (90:10) solvent flow rate was 0.5 ml/min for each sample.

FIGURE 6. Fluorescence high pressure liquid chromatograms for the USP reference standard (A) and the manufacturers' house standards (B-E) of pyrvinium pamoate. The methanol:water (90:10) solvent flow rate was 1.0 ml/min for C and 0.5 ml/min for the other samples. Injection volumes were 10 µl for A and B, 5 µl for C and D, and 2 µl for E. The numbers 1 and 2 represent the pamoate and pyrvinium moieties, respectively.

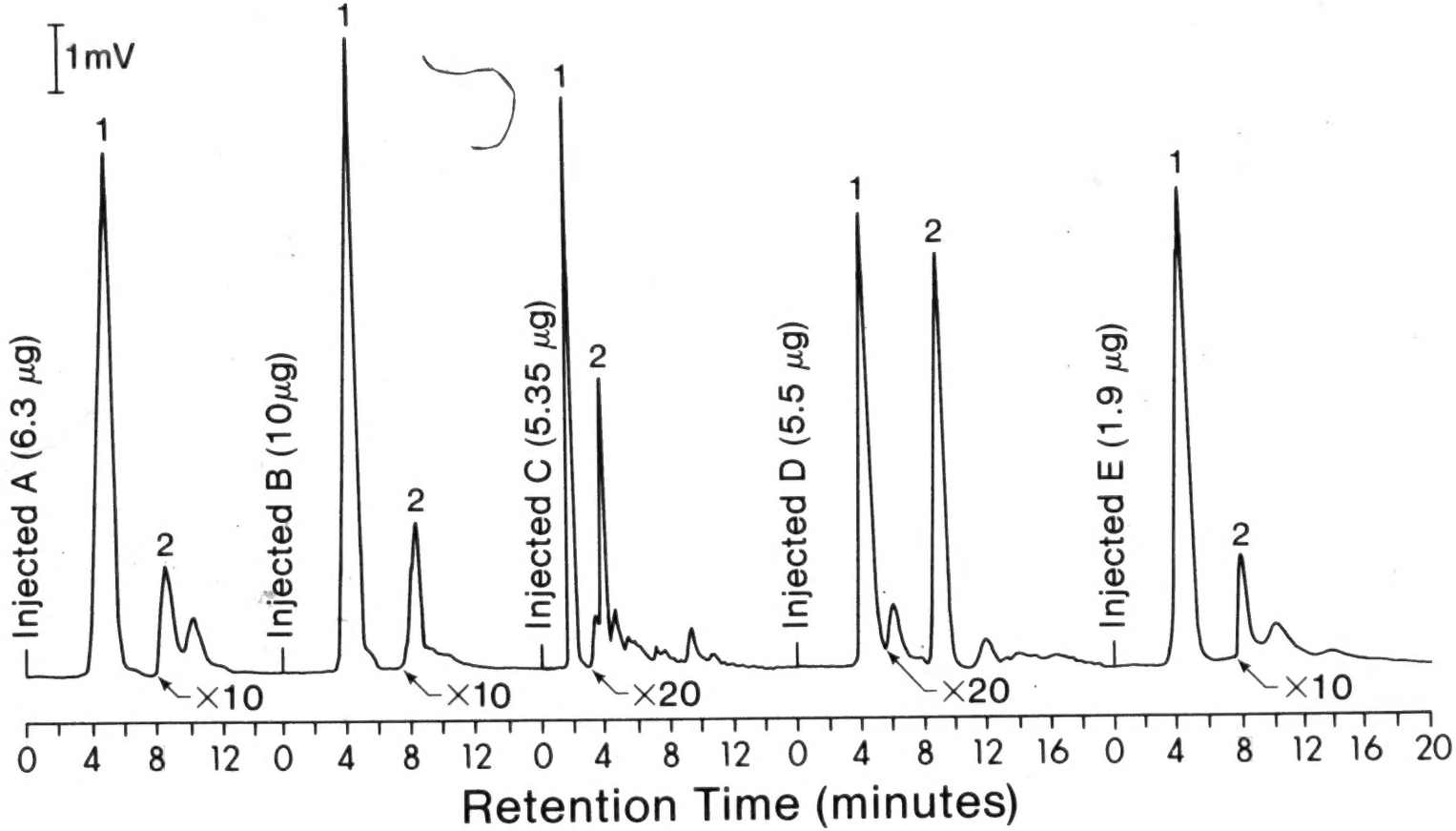
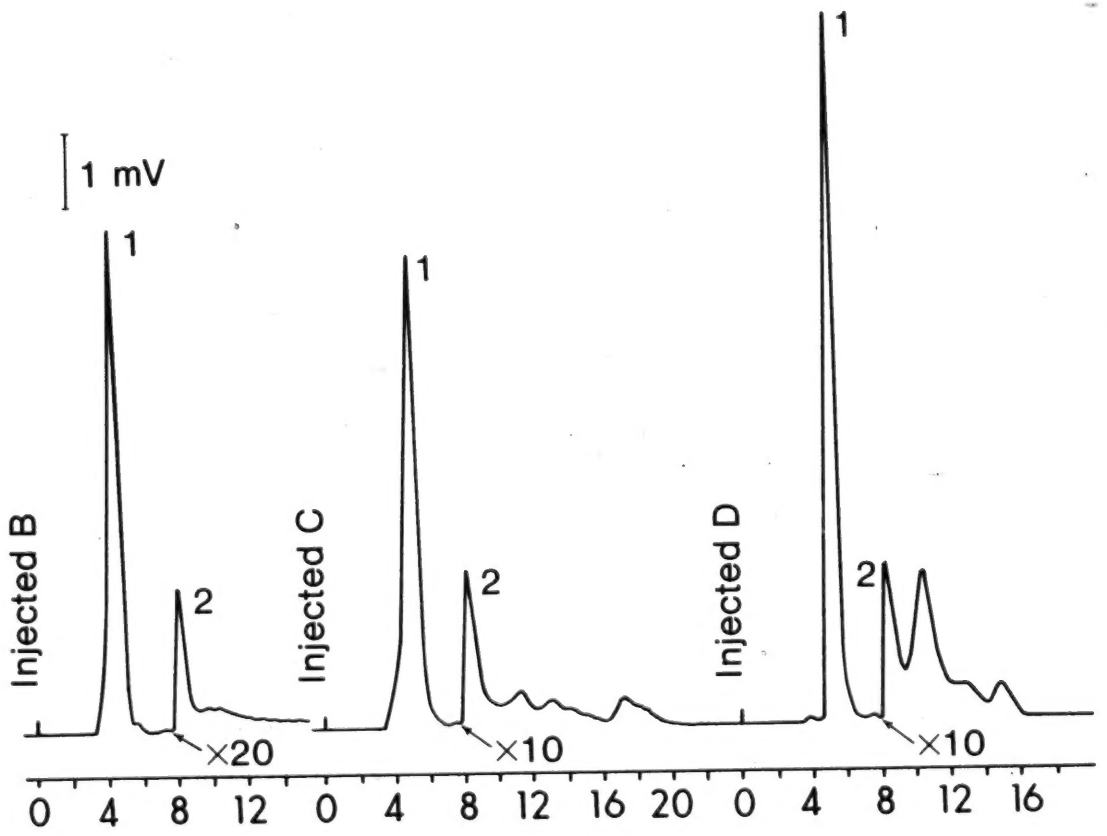

1mV
Injected A (6.3 μg)
Injected B (10μg)
Injected C (5.35 μg)
Injected D (5.5 μg)
Injected E (1.9 μg)
1
2
×10
×10
×20
×20
×10
0 4 8 12 0 4 8 12 0 4 8 12 0 4 8 12 16 0 4 8 12 16 20
Retention Time (minutes)

FIGURE 7. Fluorescence high pressure liquid chromatograms for the methanol extracts of commercially available pyrvinium pamoate tablets. The letters B, C, and D correspond to the manufacturers of the house standards in Figure 6. Injection volumes were 10 µl and the methanol:water (90:10) solvent flow rate was 0.5 ml/min for each sample. The numbers 1 and 2 represent the pamoate and pyrvinium moieties, respectively.


1 mV
1
2
×20
Injected B
1
2
×10
Injected C
1
2
×10
Injected D
0 4 8 12 0 4 8 12 16 20 0 4 8 12 16
Retention Time (minutes)

ARTICLE III

An Evaluation of the Genetic Activity of Pyrvinium Pamoate and Several Other Anthelmintic Drugs in *Saccharomyces cerevisiae*

ABSTRACT

Several anthelmintic drugs that are used routinely in oxyuriasis therapy were analyzed for potential genetic activity in a diploid mitotic recombination and gene conversion assay (strain D5 of *Saccharomyces cerevisiae*), and in a haploid yeast reversion assay (strain XV185-14C). Piperazine citrate, piperazine adipate, mebendazole, thiabendazole, and pyrantel pamoate did not appear to be active genetically in either yeast strain, whereas pyrvinium pamoate was mutagenic in strain XV185-14C and recombinogenic in strain D5.

INTRODUCTION

Pyrvinium pamoate, pyrantel pamoate, piperazine citrate, piperazine adipate, mebendazole, and thiabendazole are anthelmintic drugs that are used routinely in the treatment of pinworm infestations. All of these drugs have prescription-only status in the United States, however, the U.S. Food and Drug Administration has made a tentative decision to accept the recommendation to recategorize pyrantel pamoate as a nonprescription drug (Federal Register, 1980; Pettinato, 1982). In Canada, mebendazole and thiabendazole can be procured only with a prescription, whereas pyrvinium pamoate, pyrantel pamoate, and the piperazine salts are over-the-counter products that are available for self-medication and they can be obtained only in pharmacies. Mexico does not have an official pharmacopeia and there is ready access to most drugs, including anthelmintic drugs, for self-medication by the

general public.

Several anthelmintic drugs have been evaluated for mutagenicity in bacterial assays. Tutikawa *et al.* (1978) found pyrvinium pamoate and pyrantel pamoate to be nonmutagenic when tested in a recombination-deficient strain of *Bacillus subtilis*, however, DNA-damaging products were generated when each compound was mixed with sodium nitrite in an acidic medium at 37°C. These mixtures gave positive results for mutagenicity in several strains of *Salmonella typhimurium* and *Escherichia coli*. Since nitrites act on DNA to produce miscoding lesions (Steinberg and Thom, 1940a,b; Vielmetter and Schuster, 1960a,b; Laval and Boiteux, 1983), the mutagenicity observed in the system of Tutikawa *et al.* (1978) need not be attributed to pyrvinium pamoate and pyrantel pamoate.

MacPhee and Podger (1977) reported that pyrantel pamoate, thiabendazole, piperazine citrate, and piperazine adipate were not mutagenic in *Salmonella typhimurium* and that pyrvinium pamoate was mutagenic only when the S9 liver microsomal fraction was added to the assay. Similarly, Lake and de la Iglesia (1981) noted that pyrvinium pamoate induced mutations in *Salmonella typhimurium* only in the presence of rat or human liver microsomes.

Cortinas de Nava *et al.* (1983) tested mebendazole, pyrantel pamoate, and pyrvinium pamoate in *Salmonella typhimurium* and found that mebendazole and pyrantel pamoate were not mutagenic, but all of the batches of pyrvinium pamoate were mutagenic with and without activation by the S9 liver microsomal fraction. Similar results were obtained by Hennig *et al.* (1984), but only for two brands of pyrvinium pamoate; the remainder of the brands that were tested required metabolic transforma-

tion to be mutagenic in strains TA100 and TA98 of *Salmonella typhimurium*. The samples of pyrvinium pamoate that were tested by MacPhee and Podger (1977) and Lake and de la Iglesia (1981) required activation to be mutagenic in *Salmonella typhimurium*. A possible explanation for the contradictory results is that the genetic activity of the brands of pyrvinium pamoate that are marketed in Mexico may be due to impurities that do not require metabolic activation to be mutagenic. Evidence for impurities in medical grades of pyrvinium pamoate has been disclosed by Mehta *et al.* (1982) and Hennig *et al.* (1981, 1984).

Pyrvinium chloride, pyrvinium iodide, and several brands of pyrvinium pamoate have been tested in *Saccharomyces cerevisiae* (Mehta *et al.*, 1982; Hennig *et al.*, 1984). These studies indicated that pyrvinium salts are mutagenic in haploid yeast and increase the frequency of aberrant colonies in diploid yeast.

In the present investigation, the anthelmintic drugs that are used routinely in the treatment of infestations of the common pinworm, *Enterobius (Oxyuris) vermicularis*, were evaluated for genetic activity in *Saccharomyces cerevisiae*. Mebendazole, piperazine citrate, and pyrvinium pamoate are the products that are used most commonly for oxyuriasis therapy in humans, thus samples from several pharmaceutical companies have been tested.

MATERIALS AND METHODS

Yeast Strains

Reverse mutations were studied in the haploid strain XV185-14C of *Saccharomyces cerevisiae*. The genotype of this strain is

a *ade2-1 arg4-17 lys1-1 trp5-48 his1-7 hom3-10*;

ade2-1, *arg4-17*, *lys1-1*, and *trp5-48* are suppressible ocher nonsense mutations, *his1-7* is a missense mutation, and *hom3-10* is a putative frameshift mutation.

The genotype of the diploid strain D5 (Zimmermann, 1973) of *Saccharomyces cerevisiae* is

$$\frac{\alpha}{a}\ \frac{trp1\text{-}1}{+}\ \frac{ade2\text{-}40}{ade2\text{-}119}\ \frac{MAL1}{+}\ \frac{+}{MAL4}\ .$$

In this strain, mitotic recombination results in sectored colonies with red-pink or red-pink-white phenotypes, and red, pink, red-white, or pink-white colonies are produced as a consequence of mitotic gene conversion or mutations in the *ade2* gene.

Mutagenicity Assays

The anthelmintic drugs were used as supplied by the manufacturers: pyrvinium pamoate (Parke-Davis Canada Incorporated and Laboratorios Columbia, SA), pyrantel pamoate (Pfizer Company Limited), piperazine citrate (Burroughs Wellcome Limited and Laboratorios Kan, SA), piperazine adipate (Glaxo Laboratories, a division of Allen & Hanburys), mebendazole (Ortho Pharmaceutical [Canada] Limited and Laboratorios Columbia, SA), and thiabendazole (Merck, Sharp & Dohme Canada Limited).

Stock solutions of pyrvinium pamoate (18.4 mg/ml) were prepared in dimethylsulfoxide (DMSO) and 0.1 ml was added to the treatment tubes.

The other anthelmintic drugs have a very limited solubility in DMSO, and in aqueous media, therefore stock solutions were not prepared for piperazine citrate, piperazine adipate, mebendazole, thiabendazole, and pyrantel pamoate. Instead, the appropriate quantity of each of these compounds was added directly into the treatment tubes.

The molarities that were used in the analysis of piperazine citrate (1/3 of the molarities that were used for piperazine adipate) provided an equivalent weight of piperazinium ion (see Figure 8). This permitted a direct comparison of the active ingredient of these two piperazine salts (Tables 16 and 17).

Reversion Assay (Strain XV185-14C)

Cells that had been grown on YEPD-agar (1.0% yeast extract, 2.0% peptone, 2.0% dextrose, and 1.5% agar) for 48 hours were inoculated into liquid YEPD medium. Inocula of $1x10^7$ cells/ml and $1x10^5$ cells/ml were incubated at 30°C to achieve stationary- and logarithmic-phase cells, respectively. For the mutagenicity assays, which have been described by Mehta *et al.* (1982), cell suspensions ($5x10^7$ - $1x10^8$ cells/ml) of each yeast culture were prepared in phosphate buffer (pH 7.0).

The treatment tubes, which contained the appropriate quantity of each drug plus 0.1 ml of DMSO, and the control tubes, which contained 0.1 ml of DMSO, each received 3.9 ml of cell suspension. These reaction mixtures were incubated at 30°C in a waterbath shaker. At several time intervals, cell samples were removed, washed in 50 ml of 2.5% DMSO by centrifugation, and resuspended in 4.0 ml of buffer. Undiluted samples were plated onto the appropriate omission media (MC minus arginine, MC

minus tryptophan, MC minus histidine, and MC minus threonine), which have been described by von Borstel *et al.* (1971), and incubated at 30°C for 4-6 days. Diluted samples were spread onto YEPD-agar plates for the survival estimation.

Mitotic Recombination and Gene Conversion Assay (Strain D5)

The procedures for this assay were similar to those described for the haploid reversion assay except that initial inocula of $1x10^4$ cells/ml and $1x10^6$ cells/ml were used to grow logarithmic- and stationary-phase cells, respectively. Diluted samples were plated onto YEPD-agar, which contains a low concentration of adenine, and incubated at 30°C for 7-10 days. These plates were then scored for survival and the color development of adenine-dependent colonies.

RESULTS AND DISCUSSION

The apparent enhancement of cell viability that is suggested by the survival data in Tables 14-17 is an artefact caused by the semi-solid drug matrix that is precipitated during the centrifugation process (Mehta *et al.*, 1982). This apparent enhancement of cell survival is not real and is characteristic of drugs that have a limited solubility in aqueous media.

Due to their limited solubility, these anthelmintic drugs were weighed directly into the treatment tubes. This resulted in saturated solutions and the yeast cells should have absorbed the drugs to their maximum capacity. In addition, the lipophilic solvent DMSO was used to

solubilize as much of each anthelmintic drug as possible, and to enhance the absorption of the drugs by facilitating their passage across cell membranes.

Since pyrvinium pamoate has been demonstrated to be a mutagen, it served as a positive control; DMSO (5.0% and 2.5%) and water served as the negative controls. As is evident from the reversion frequencies in Table 15, 5.0% DMSO does not produce a significant increase in revertants when it is compared to water, although a decrease in survival was observed.

Mebendazole, thiabendazole, pyrantel pamoate, piperazine citrate, and piperazine adipate did not exhibit genetic activity in either stationary- or logarithmic-phase cells of yeast (Tables 14-17). The frequencies of aberrant colonies were not increased significantly in strain D5 (Tables 14 and 16), nor did these anthelmintic drugs produce significant increases in the frequencies of revertants in strain XV185-14C (Tables 15 and 17). Also, there was no apparent difference between the Canadian and Mexican samples of mebendazole and piperazine citrate (Tables 14-17).

Piperazine citrate, piperazine adipate, and pyrantel pamoate do not appear to be toxic to either stationary- or logarithmic-phase cells of yeast but mebendazole, thiabendazole, and pyrvinium pamoate caused a decrease in survival at 24 hours and 48 hours (Tables 14-17). The toxicity of pyrvinium pamoate is already evident after 3 hours of exposure (Tables 16 and 17).

Pyrvinium pamoate exhibited genetic activity in both stationary- and logarithmic-phase cells of *Saccharomyces cerevisiae*. In strain D5, the frequencies of aberrant colonies (mitotic recombination, gene

conversion, and mutation) were increased significantly over the control levels (Tables 14 and 16). There appears to be little difference between the Canadian and Mexican samples of pyrvinium pamoate when they are compared on the basis of their degree of genetic activity in strain D5, however, the Mexican sample is much more toxic, as is evident from the survival data at 24 hours and 48 hours of exposure to the drug (Tables 14 and 16).

Only in the reversion assay with strain XV185-14C was there a marked difference between the Canadian and Mexican samples of pyrvinium pamoate. This anthelmintic drug is a potent mutagen and it induces reversions of missense, nonsense, and frameshift alleles. At 3 hours and 24 hours of exposure of logarithmic-phase cells to the Canadian sample of pyrvinium pamoate (Table 17), there was an approximately 3-fold increase in revertant frequencies (*HIS*$^+$, *TRP*$^+$, and *HOM*$^+$), whereas the Mexican sample (Table 15) induced 10- to 30-fold increases in revertant frequencies (*HIS*$^+$, *ARG*$^+$, and *HOM*$^+$) when stationary-phase cells of strain XV185-14C were exposed to the drug for 24 hours.

The pyrvinium moiety is responsible for the mutagenicity of pyrvinium pamoate, as has been demonstrated with monopyrvinium salts (Mehta *et al.*, 1982). Lake and de la Iglesia (1981) reported that pamoic acid was not mutagenic in *Salmonella typhimurium* and Mehta *et al.* (1982) found monopotassium pamoate to be nonmutagenic in *Saccharomyces cerevisiae* (strains D5 and XV185-14C).

In the yeast assays, the S9 microsomal fraction was not required to elicit a mutagenic response to pyrvinium pamoate since *Saccharomyces cerevisiae* possesses cytochrome P-450 activity comparable to the mixed-function oxygenase system (also referred to as the polysubstrate

monooxygenase system) in mammalian liver (Callen and Philpot, 1977). Logarithmic-phase cells of the diploid strain D5, when they have been grown on a glucose-containing medium, have about 10X more cytochrome P-450 content than logarithmic-phase cells of the haploid strain XV185-14C that have been grown under the same conditions (D.F. Callen, personal communication; U.G.G. Hennig and D.F. O'Connell, unpublished data).

Pyrvinium pamoate is not absorbed systemically (Rollo, 1980), thus this drug should not undergo metabolism in the liver, where the activation of a promutagen to a mutagen is expected to take place. Studies in mice and humans have demonstrated that mutagenic metabolites do not appear in the urine after a single, therapeutic dose of pyrvinium pamoate (Lake and de la Iglesia, 1981; Cortinas de Nava *et al.*, 1983); the 24-hour urine samples were tested in *Salmonella typhimurium*. These assays indicate that pyrvinium pamoate is not absorbed systemically in mammals and thus it cannot be metabolized by the hepatic microsomal enzymes. A localized biotransformation by intestinal microflora would be the only source of mutagenic metabolites of pyrvinium pamoate.

The unexpected mutagenic response to pyrvinium pamoate that was obtained without activation by the S9 fraction (Cortinas de Nava *et al.*, 1983; Hennig *et al.*, 1984) can be postulated to be due to impurities in the medical grades of the drug. Interestingly, these mutagenic components did not appear in the 24-hour urine samples of the treated mice (Cortinas de Nava *et al.*, 1983).

Bellander *et al.* (1984) have reported that the anthelmintic drug piperazine is nitrosated rapidly *in vitro*. *In vivo*, the suspected carcinogen *N*-mononitrosopiperazine was found in the gastric juice after

30 minutes, and in the 3-hour urine samples, when a therapeutic dose of piperazine was administered to fasting, healthy volunteers. The carcinogen *N,N'*-dinitrosopiperazine was not detected. The concomitant administration of an antioxidant, ascorbic acid (vitamin C), resulted in a significant inhibition of the nitrosation of piperazine.

The mechanism of anthelmintic action of the piperazine salts and pyrantel pamoate is the production of a neuromuscular blockade in the parasite; this is an effect that is also exerted on the host and therefore these agents can be administered only by the oral route. The piperazine salts are absorbed readily from the gastrointestinal tract and up to 15% of a therapeutic dose of pyrantel pamoate is absorbed systemically (Rollo, 1980), however, both piperazine and pyrantel pamoate cause neurological side effects that are characterized by headaches and dizziness. This is especially critical in individuals with renal dysfunction, and young children, who are the intended consumers of anthelmintic drugs, have compromised kidney function.

About 10% of a single, therapeutic dose of mebendazole is absorbed systemically (Rollo, 1980) and it causes more severe gastrointestinal side effects than any of the other anthelmintic drugs (The Medical Letter, 1982). Pyrvinium pamoate is mutagenic. The consequences of untreated, or inadequately treated, pinworm infestations can include the progression of the infestation into the bladder and, more commonly, secondary bacterial and/or fungal infections. When these potential consequences, as well as the risks and benefits of all of the anthelmintic drugs are considered, pyrvinium pamoate, although mutagenic, should not be excluded as an agent in oxyuriasis therapy.

TABLE 14

Induction of aberrant colonies in stationary-phase cells of strain D5 of *Saccharomyces cerevisiae* after treatment with several anthelmintic drugs

Anthelmintic[a] and Dose (moles/l)	Exposure Time (hours)	Survival (%)	Total Colonies[b]	Total Aberrant Colonies[c] (%)
control	0	100	2918	0.07 (2)
5.0% DMSO	3	100	3981	0.02 (1)
	24	100	3365	<0.03 (0)
	48	100	3945	0.05 (2)
pyrvinium pamoate	0	175	4934	0.59 (29)
0.0004	3	152	5638	2.43 (137)
	24	91	3063	1.9 (57)
	48	29	1127	1.6 (18)
piperazine citrate	0	89	1732	<0.06 (0)
0.02	3	72	1901	<0.05 (0)
	24	84	1882	0.05 (1)
	48	66	1745	<0.06 (0)
mebendazole	0	150	2918	<0.03 (0)
0.008	3	144	3830	<0.03 (0)
	24	136	3056	0.03 (1)
	48	73	1918	<0.05 (0)

[a] Pyrvinium pamoate and mebendazole were supplied by Laboratorios Columbia, SA (Mexico), and piperazine citrate was supplied by Laboratorios Kan, SA (Mexico).

[b] The total number of surviving colonies on a complete medium (YEPD-agar) that contains a low concentration of adenine.

[c] Includes red-pink, red-pink-white, red, red-white, pink, and pink-white colonies. The figures in parentheses constitute the total number of aberrant colonies, as determined by plate counts.

TABLE 15

Reversion frequencies induced in stationary-phase cells of strain XV185-14C of *Saccharomyces cerevisiae* after treatment with several anthelmintic drugs

Anthelmintic[a] and Dose (moles/l)	Survival[b] (%)		Reversion Frequencies[c] (revertants/10^7 survivors)					
			HIS$^+$		*ARG*$^+$		*HOM*$^+$	
	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
controls								
5.0% water	100 (112)	100 (171)	21 (94)	17 (115)	13 (60)	8 (52)	2 (10)	3 (18)
5.0% DMSO	70 (79)	87 (149)	34 (107)	17 (102)	25 (80)	11 (68)	6 (19)	3 (20)
pyrvinium pamoate								
0.0002	120 (135)	16 (28)	46 (247)	374 (419)	18 (96)	160 (179)	5 (26)	28 (31)
0.0004	88 (99)	9 (16)	45 (180)	483 (309)	19 (75)	161 (103)	3 (12)	39 (25)
piperazine citrate								
0.01	73 (82)	117 (200)	27 (89)	16 (132)	23 (75)	6 (52)	5 (16)	2 (16)
0.02	97 (109)	113 (194)	23 (99)	17 (132)	18 (80)	5 (21)	3 (15)	2 (8)
mebendazole								
0.005	171 (192)	69 (118)	9 (71)	33 (157)	6 (48)	9 (43)	2 (16)	1 (5)
0.098	143 (160)	119 (204)	11 (70)	13 (107)	9 (59)	8 (65)	3 (19)	2 (16)

[a] Pyrvinium pamoate and mebendazole were supplied by Laboratorios Columbia, SA (Mexico), and piperazine citrate was supplied by Laboratorios Kan, SA (Mexico).

[b] The figures in parentheses constitute the total number of surviving colonies on 2 plates of YEPD-agar.

[c] The figures in parentheses constitute the total number of revertants, as determined by plate counts.

TABLE 16

Induction of aberrant colonies in logarithmic-phase cells of strain D5 of *Saccharomyces cerevisiae* after 3-hour and 24-hour treatments with several anthelmintic drugs

Anthelmintic[a] and Dose (moles/l)	Survival (%)		Total Colonies[b]		Total Aberrant Colonies[c] (%)	
	3 hr	24 hr	3 hr	24 hr	3 hr	24 hr
control						
2.5% DMSO	100	100	2408	2136	0.1 (3)	0.4 (8)
pyrvinium pamoate						
0.0004	86	86	2059	1844	0.3 (6)	2.7 (49)
piperazine citrate						
0.0035	105	102	2527	2176	0.1 (3)	0.2 (4)
0.0070	83	108	2010	2300	0.2 (3)	0.48(11)
0.0105	95	114	2281	2186	0.2 (5)	0.2 (4)
0.0140	106	95	2556	2043	0.2 (5)	0.3 (6)
piperazine adipate						
0.01	89	126	2151	2693	0.2 (5)	0.2 (6)
0.02	113	88	2731	1891	0.2 (5)	0.1 (2)
0.03	103	90	2485	1922	0.08(2)	0.1 (2)
0.04	83	106	1990	2261	0.2 (4)	0.2 (5)
mebendazole						
0.01	95	78	2298	1659	0.3 (7)	0.1 (2)
0.02	101	69	2439	1471	0.2 (4)	0.3 (5)
0.03	105	80	2527	1725	0.2 (5)	0.4 (6)
0.04	75	80	1811	1714	0.1 (2)	0.2 (4)

TABLE 16 (continued)

Anthelmintic[a] and Dose (moles/l)	Survival (%)		Total Colonies[b]		Total Aberrant Colonies[c] (%)	
	3 hr	24 hr	3 hr	24 hr	3 hr	24 hr
thiabendazole						
0.01	106	74	2555	1586	<0.04 (0)	0.4 (7)
0.02	81	81	1952	1738	0.2 (3)	0.2 (4)
0.03	86	70	2084	1487	0.2 (4)	0.2 (3)
0.04	120	56	2878	1204	0.2 (7)	0.2 (2)
pyrantel pamoate						
0.005	102	94	2470	2010	0.53(13)	0.2 (3)
0.010	114	120	1645	2560	0.06 (1)	<0.04 (0)
0.020	97	121	2336	2585	0.1 (3)	0.04 (1)
0.030	98	163	2348	3476	0.3 (7)	0.06 (2)
0.040	105	126	2529	2697	0.3 (8)	0.04 (1)

[a]Pyrvinium pamoate (Parke-Davis Canada Incorporated), piperazine citrate (Burroughs Wellcome Limited), piperazine adipate (Glaxo Laboratories, a division of Allen & Hanburys), mebendazole (Ortho Pharmaceutical [Canada] Limited), thiabendazole (Merck, Sharp & Dohme Canada Limited), and pyrantel pamoate (Pfizer Company Limited).

[b]The total number of surviving colonies on a complete medium (YEPD-agar) that contains a low concentration of adenine.

[c]Includes red-pink, red-pink-white, red, red-white, pink, and pink-white colonies. The figures in parentheses constitute the total number of aberrant colonies, as determined by plate counts.

TABLE 17

Reversion frequencies induced in logarithmic-phase cells of strain XV185-14C of *Saccharomyces cerevisiae* after 3-hour and 24-hour treatments with several anthelmintic drugs

Anthelmintic[a] and Dose (moles/l)	Survival[b] (%)		Reversion Frequencies[c] (revertants/10^7 survivors)					
			HIS$^+$		*TRP*$^+$		*HOM*$^+$	
	3 hr	24 hr	3 hr	24 hr	3 hr	24 hr	3 hr	24 hr
control								
2.5% DMSO	100 (1876)	100 (1433)	16 (47)	16 (37)	15 (45)	25 (58)	2 (13)	2 (8)
pyrvinium pamoate								
0.0004	93 (1741)	80 (1143)	56(155)	43 (79)	43(120)	45 (82)	4 (23)	8 (30)
piperazine citrate								
0.0035	87 (1626)	104 (1483)	20 (51)	16 (37)	23 (60)	17 (41)	3 (14)	1 (5)
0.0070	90 (1694)	117 (1683)	19 (52)	16 (43)	24 (66)	16 (42)	2 (13)	1 (7)
0.0105	89 (1683)	129 (1845)	20 (53)	16 (46)	22 (58)	18 (52)	2 (13)	1 (7)
0.0140	111 (2088)	126 (1799)	18 (59)	20 (59)	18 (60)	17 (49)	1 (9)	1 (7)
piperazine adipate								
0.01	56 (1047)	112 (1604)	17 (29)	11 (28)	25 (42)	14 (37)	1 (2)	1 (6)
0.02	72 (1353)	109 (1557)	17 (36)	14 (35)	18 (38)	14 (36)	2 (7)	1 (3)
0.03	94 (1770)	114 (1632)	18 (52)	18 (48)	19 (54)	17 (45)	2 (10)	1 (3)
0.04	98 (1828)	129 (1849)	17 (49)	14 (41)	17 (51)	13 (38)	3 (18)	2 (11)

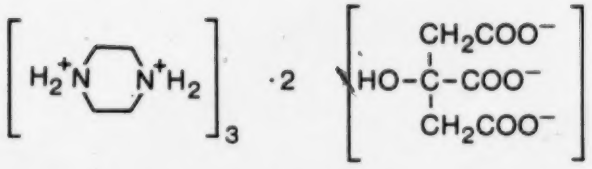
TABLE 17 (continued)

Anthelmintic[a] and Dose (moles/l)	Survival[b] (%)		Reversion Frequencies[c] (revertants/10^7 survivors)					
			HIS+		*TRP*+		*HOM*+	
	3 hr	24 hr	3 hr	24 hr	3 hr	24 hr	3 hr	24 hr
mebendazole								
0.01	76 (1417)	98 (1406)	30 (67)	14 (31)	25 (57)	15 (34)	1 (5)	1 (6)
0.02	78 (1471)	95 (1361)	22 (51)	15 (32)	26 (62)	19 (41)	1 (5)	<1 (1)
0.03	105 (1966)	102 (1464)	21 (67)	13 (31)	25 (78)	14 (34)	2 (11)	1 (4)
0.04	106 (1994)	83 (1183)	24 (75)	13 (25)	19 (61)	16 (31)	1 (8)	1 (5)
thiabendazole								
0.01	86 (1602)	64 (911)	24 (62)	11 (16)	28 (72)	9 (13)	2 (10)	1 (4)
0.02	122 (2288)	56 (795)	16 (60)	11 (14)	16 (60)	14 (18)	2 (11)	1 (2)
0.03	82 (1542)	77 (1106)	17 (41)	7 (13)	17 (41)	9 (16)	1 (7)	3 (12)
0.04	100 (1868)	72 (1024)	12 (37)	2 (3)	16 (48)	7 (11)	1 (7)	1 (3)
pyrantel pamoate								
0.01	105 (1964)	81 (1163)	23 (73)	22 (40)	26 (81)	18 (34)	2 (14)	1 (4)
0.02	94 (1765)	102 (1458)	30 (84)	15 (36)	27 (77)	19 (45)	3 (15)	6 (30)
0.03	78 (1460)	93 (1333)	26 (60)	15 (32)	32 (74)	19 (41)	2 (8)	1 (6)
0.04	116 (2181)	67 (959)	25 (88)	19 (29)	16 (57)	20 (30)	1 (4)	9 (27)

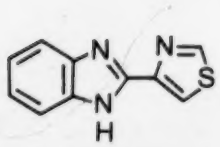
[a]Pyrvinium pamoate (Parke-Davis Canada Incorporated), piperazine citrate (Burroughs Wellcome Limited), piperazine adipate (Glaxo Laboratories, a division of Allen & Hanburys), mebendazole (Ortho Pharmaceutical [Canada] Limited), thiabendazole (Merck, Sharp & Dohme Canada Limited), and pyrantel pamoate (Pfizer Company Limited).

[b]The figures in parentheses constitute the total number of surviving colonies on 4 plates of YEPD-agar.

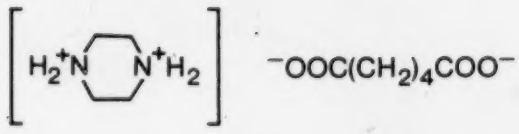
[c]The figures in parentheses constitute the total number of revertants, as determined by plate counts.



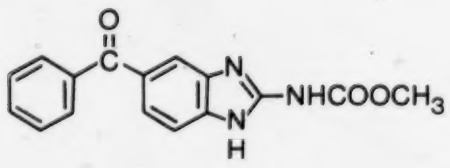
piperazine citrate
(MW 642.68)



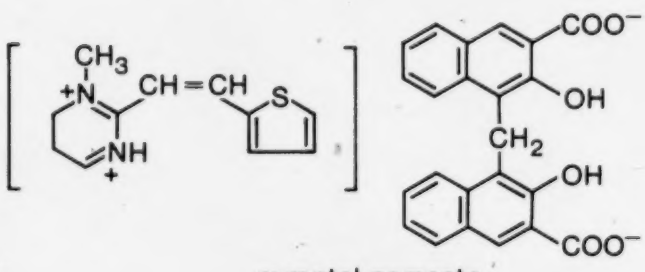
thiabendazole
(MW 201.26)



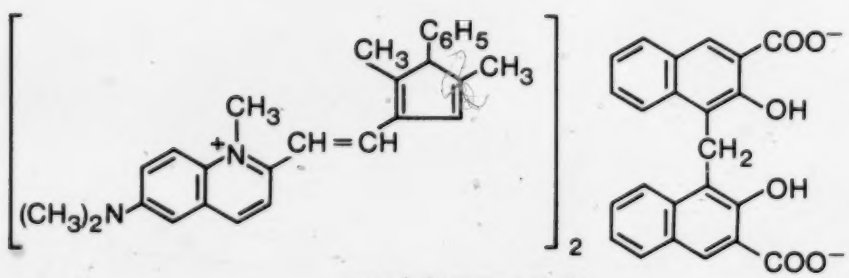
piperazine adipate
(MW 232.28)



mebendazole
(MW 295.30)



pyrantel pamoate
(MW 594.68)



pyrvinium pamoate
(MW 1151.44)

FIGURE 8. The structural formulas and the molecular weights of the anthelmintic drugs that are used routinely in oxyuriasis therapy in humans.

ARTICLE IV

An Analysis of the Structure-Activity Relationships Involved in the Mutagenicity of Pyrvinium Salts in *Saccharomyces cerevisiae:* A Comparison of the Genetic Activity of Analogs

ABSTRACT

An investigation of the structural requirements for the mutagenic action of pyrvinium pamoate and pyrvinium iodide has been carried out using two different analogs of the monopyrvinium salt, pyrvinium iodide. The genetic activity of the analogs was studied in diploid and haploid yeast (strains D5, D7, XV185-14C, XY718-1A, and 7854-1A of *Saccharomyces cerevisiae*) in order to gain information about the structural features that are required for the genetic activity of pyrvinium. Substitution with a methyl group at the 6-position of pyrvinium iodide did not affect the mutagenicity. In comparison to pyrvinium iodide, the methyl-analog is of equal toxicity and it induces frameshift and base-substitution mutations just as readily. In contrast, the methyl-analog induces transitions and transversions, whereas pyrvinium iodide only induces significant levels of transitions. With the 6-chloro-analog, both the toxicity and the mutagenicity were diminished, however, it induced transitions and frameshift mutations at frequencies similar to the dipyrvinium salt, pyrvinium pamoate, which is less accessible to the yeast cells because it does not dissociate as readily as pyrvinium iodide.

INTRODUCTION

Pyrvinium pamoate is mutagenic in bacteria and yeast. The drug induces the reversion of nonsense, missense, and frameshift alleles in haploid yeast (strain XV185-14C). In strains XY718-1A and 7854-1A of *Saccharomyces cerevisiae*, frameshift suppressor mutations and reversions of the frameshift alleles (*his4-519*, *his4-38*, and *leu2-3*) are induced by pyrvinium pamoate. Relatively pure medical grades of this anthelmintic drug predominantly induce transitions in strain XV185-14C of *Saccharomyces cerevisiae*. The reversion frequencies that were obtained for two ΦX174 amber codons in an *in vitro* DNA replication system that is mediated by T4 bacteriophage replication proteins also suggest that pyrvinium pamoate primarily induces transitions (Razzaki *et al.*, 1984). In diploid yeast (strain D5 of *Saccharomyces cerevisiae*), 50-75% of the aberrant colonies that are induced by pyrvinium pamoate are mitotic recombinants and the remainder can arise from other genetic events, such as mutations, gene conversion, or chromosome loss (Mehta *et al.*, 1982; Hennig *et al.*, 1984).

The genetic activity varies considerably among the medical grades of pyrvinium pamoate that have been tested in yeast. The enhanced genetic activity of some of these medical grades has been attributed to monopyrvinium salts, which may be present as incomplete reactants of the synthetic process used in producing pyrvinium pamoate (Hennig *et al.*, 1984). Pyrvinium chloride and pyrvinium iodide, which are monopyrvinium salts, are potent mutagens in haploid and diploid yeast and, since monopotassium pamoate is nontoxic and nonmutagenic in strains XV185-14C and D5 of *Saccharomyces cerevisiae*, the pyrvinium

moiety is responsible for the genetic activity of pyrvinium pamoate (Mehta *et al.*, 1982). The monopyrvinium salts are more mutagenic than pyrvinium pamoate and this is probably due to their smaller molecular size (see Figures 2 and 9), and to the fact that they can undergo ionization to a much greater extent. This increases the availability of pyrvinium molecules for cellular uptake (Mehta *et al.*, 1982).

To eliminate the impurities in pyrvinium pamoate, and to determine the effect of different functional groups on the mutagenic action of this drug, the custom-synthesis of pyrvinium pamoate, pyrvinium iodide, and two analogs of pyrvinium iodide was arranged. The difficulty in removing the incomplete reactants (monopyrvinium salts) during the purification of pyrvinium pamoate has been corroborated by Lancaster Synthesis Limited (E. Wildsmith, Director, personal communication).

The prototype for chemicals that are carcinogens in animals is 2-aminofluorene (Figure 1). This compound and a metabolite, 2-acetylaminofluorene, were not mutagenic in *Salmonella typhimurium* unless the modified bacterial assay, which incorporates mammalian liver microsomes (the S9 fraction) into the system, was used (Ames *et al.*, 1972; McCann *et al.*, 1975). The genetic activity of 2-aminofluorene has been attributed to the free, aromatic amino-substituent, which is *N*-acetylated and *N*-hydroxylated by hepatic enzymes; *N*-hydroxy-2-acetylaminofluorene undergoes sulfate conjugation, mediated by sulfotransferases, to the ultimate carcinogen that is capable of reacting with protein, RNA, and DNA.

Pyrvinium salts have an aromatic, dimethylamino-substituent at the 6-position (Figure 9), which can be demethylated by mammalian liver enzymes and, therefore, these drugs have the potential for further

activation of the type that occurs with 2-aminofluorene and 2-acetylaminofluorene. The replacement of this dimethylamino-substituent with either a chloro- or a methyl-substituent was carried out to assess the effect that substitution at the 6-position has on the mutagenicity. An abolishment of the mutagenicity would suggest that the 6-position is the only active site on the pyrvinium molecule, whereas decreased or residual mutagenicity would mean that the molecule has more than one active center.

MATERIALS AND METHODS

Pyrvinium Salts and Analogs

Pyrvinium pamoate, pyrvinium iodide, and the 6-chloro- and 6-methyl-analogs of pyrvinium pamoate were custom-synthesized by Lancaster Synthesis Limited (Eastgate, Lancashire, England). The analogs have chloro- and methyl-substituents in place of the 6-dimethylamino-substituent of pyrvinium iodide (Figure 9). Stock solutions of pyrvinium pamoate (18.2 mg/ml), pyrvinium iodide (8.2 mg/ml), the 6-chloro-analog (16.0 mg/ml), and the 6-methyl-analog (15.4 mg/ml) were prepared in dimethylsulfoxide (DMSO); when required, the stock solutions were diluted with DMSO.

Yeast Strains

The haploid strains (XV185-14C, genotype a *ade2-1 arg4-17 lys1-1 trp5-48 his1-7 hom3-10*; XY718-1A, genotype a *his4-519 leu2-3*; and 7854-1A, genotype a *his4-38 leu2-3*) and the diploid strains

(D5, genotype $\frac{\alpha}{a}$ $\frac{trp1\text{-}1}{+}$ $\frac{ade2\text{-}40}{ade2\text{-}119}$ $\frac{MAL1}{+}$ $\frac{+}{MAL4}$ and D7, genotype $\frac{\alpha}{a}$ $\frac{ade2\text{-}40}{ade2\text{-}119}$ $\frac{trp5\text{-}12}{trp5\text{-}27}$ $\frac{ilv1\text{-}92}{ilv1\text{-}92}$) of *Saccharomyces cerevisiae* were grown on YEPD-agar (1.0% yeast extract, 2.0% peptone, 2.0% dextrose, and 1.5% agar) that was supplemented with extra amino acids plus adenine and uracil, at 30°C for 48 hours. These cells were inoculated into a culture medium that consisted of liquid YEPD supplemented with extra amino acids plus adenine. The cultures were incubated for 15-17 hours in a waterbath shaker at 30°C to attain stationary-phase cells. The cells were harvested by centrifugation, washed in 0.1 M sodium phosphate buffer (pH 7.0), and cell suspensions ($5x10^7$ - $1x10^8$ cells/ml) of each yeast strain were prepared in buffer.

Mutagenicity Assays

Detailed procedures for the mutagenicity assays that utilize yeast have been described elsewhere (von Borstel *et al.*, 1981c). Stationary-phase cells of yeast (the buffered cell suspensions of strains XV185-14C, XY718-1A, 7854-1A, D5, and D7) were dispensed into test tubes (3.9 ml/tube). In addition, the control tubes received 0.1 ml of DMSO and the treatment tubes received 0.1 ml of a stock solution, or a dilution of a stock solution, of a pyrvinium salt or an analog. The tubes were incubated at 30°C in a waterbath shaker. Cell samples (1.2 ml) were taken at 3-, 8-, and 24-hour intervals, washed twice in phosphate buffer, and resuspended in 4.0 ml (strains XV185-14C, D5, and D7) or 4.5 ml (strains XY718-1A and 7854-1A) of phosphate buffer.

For the survival estimation, serial dilutions were prepared in buffer and plated onto a complete medium, such as Mortimer complete (MC)

or YEPD, or onto MC with limiting adenine (5 μg/ml), which permits the color development of adenine-dependent colonies (strains D5 and D7); the plates were incubated at 30°C for 3-7 days. Undiluted samples were plated onto the appropriate omission media to obtain revertants. These plates were incubated for 6 days (strains D7 and XV185-14C) or 14 days (strains XY718-1A and 7854-1A). The details for the preparation of the media have been described by von Borstel *et al.* (1971).

MC minus arginine with limiting adenine (5 μg/ml) was the medium used to distinguish between events at the *arg4* locus (pink revertants) and events at the nonsense (ocher) suppressor loci (white revertants) in strain XV185-14C. Coreversion analyses of the HIS^+ and LEU^+ revertants of strains XY718-1A and 7854-1A were carried out by replica-plating to detect locus events (His^+Leu^- or His^-Leu^+ phenotypes) and frameshift suppressor events (His^+Leu^+ phenotypes).

RESULTS

The presence of the chloro-substituent at the 6-position of the chloro-analog of pyrvinium iodide was confirmed with mass spectrometry (Associated Electrical Industries MS9) by positive fast atom bombardment, using glycerol/sulfolane and direct probe analysis. The mass spectral information obtained for the molecular ions of the two most abundant isotopes of chlorine is listed as the exact mass and the relative abundance (M^+Cl^{37}, 375.145190, 35.75% and M^+Cl^{35}, 373.147153, 100.00%); the exact mass corresponds to that expected for the 6-chloro-analog of pyrvinium iodide (Figure 9).

Proton magnetic resonance spectrometry (Bruker WH-200 nuclear resonance spectrometer) also confirmed the identity of the functional groups at the 6-position for pyrvinium iodide and the chloro- and methyl-analogs. The concentration of each sample was about 8-10 mg/ml DMSO-D_6, the tubes had an outside diameter of 5 mm, and DMSO-D_6 was used as the internal standard. The integration patterns and chemical shifts correspond to those expected (Figure 9) for pyrvinium iodide (5'-CH_3 at 2.04 ppm, 2'-CH_3 at 2.27 ppm, DMSO-D_6 at 2.54 ppm, 6-$N(CH_3)_2$ at 3.15 ppm, and *N*-CH_3 at 4.39 ppm), the 6-chloro-analog of pyrvinium iodide (5'-CH_3 at 2.03 ppm, 2'-CH_3 at 2.30 ppm, DMSO-D_6 at 2.52 ppm, and *N*-CH_3 at 4.85 ppm), and the 6-methyl-analog of pyrvinium iodide (5'-CH_3 at 2.05 ppm, 2'-CH_3 at 2.32 ppm, DMSO-D_6 at 2.54 ppm, 6-CH_3 at 2.60 ppm, and *N*-CH_3 at 4.44 ppm). In addition, the spin-spin coupling constants for the α- and β-hydrogens of the vinyl bond in each of these 3 compounds fall into the range of 13-18 cps (J. Giziewicz, personal communication), therefore they are *trans*-isomers (see Figure 9).

Pyrvinium iodide induces high levels of aberrant colonies in strains D5 and D7 of *Saccharomyces cerevisiae* (Tables 18 and 19); about half of these are mitotic recombinants. At the same dose or at lower doses, the monopyrvinium salt, pyrvinium iodide, is much more active genetically than the dipyrvinium salt, pyrvinium pamoate. The 6-methyl-analog of pyrvinium iodide, when compared to pyrvinium iodide, is equal in toxicity and it induces equivalent levels of aberrant colonies in strains D5 and D7, however, this analog appears to cause only half as much mitotic recombination as pyrvinium iodide. In comparison to pyrvinium pamoate, pyrvinium iodide, and the 6-methyl-analog

of pyrvinium iodide, the 6-chloro-analog causes fewer aberrant colonies and it is less recombinogenic in strains D5 and D7; as the dose and/or toxicity increases, the genetic activity decreases. At the lower doses, the levels of aberrant colonies induced by the 6-chloro-analog of pyrvinium iodide approach those induced by pyrvinium pamoate.

The pyrvinium salts and the analogs also cause intragenic recombination in strain D7. Pyrvinium pamoate is the least active, the 6-methyl-analog is the most active, and pyrvinium iodide is more active than the 6-chloro-analog in inducing intragenic recombination. At the higher doses of the 6-chloro-analog of pyrvinium iodide ($4x10^{-4}$ M and $8x10^{-4}$ M), the frequency of *TRP*$^+$ colonies drops below the spontaneous level (Table 19).

DMSO does not appear to have a significant effect on the spontaneous *ARG*$^+$ reversion frequency in strain XV185-14C of *Saccharomyces cerevisiae* (Table 20). Pyrvinium pamoate does not increase the frequencies of suppressor events and the frequencies of locus events drop to approximately half of the spontaneous levels. This suggests that the base-substitution mutations induced by pyrvinium pamoate are not transversions. Pyrvinium iodide induced significant increases in locus events (AT→TA and/or AT→CG transversions) at 3 hours of exposure, but at 8 hours and 24 hours, the frequencies of locus events were less than the spontaneous levels. Pyrvinium iodide induces suppressor events (GC→TA transversions), but only at 2-fold increases over the controls. The 6-chloro-analog of pyrvinium iodide does not appear to cause transversions in strain XV185-14C but it decreased the frequencies of locus events to less than the spontaneous levels, at each dose and at each exposure time (Table 20). At the highest dose ($8x10^{-4}$ M) there is some

evidence that the frequencies of suppressor events is declining gradually to less than the control levels. The 6-methyl-analog of pyrvinium iodide induced significant increases in locus (AT→TA and/or AT→GC transversions) and suppressor (GC→TA transversions) events, although suppressor events predominate.

HIS$^+$ reversion frequencies of nearly 400-fold were induced in strain XV185-14C by pyrvinium iodide, and the 6-methyl-analog is almost as mutagenic (Table 21). Pyrvinium pamoate is weakly mutagenic at 3 hours and 8 hours (only 2-fold increases in *HIS*$^+$ reversion frequencies), however, 10-fold increases were observed after 24 hours of exposure. The 6-chloro-analog of pyrvinium iodide is more mutagenic than pyrvinium pamoate, with about 6-fold increases in *HIS*$^+$ reversion frequencies. Pyrvinium pamoate does not induce significant increases in the *TRP*$^+$ reversion frequencies, whereas pyrvinium iodide induces up to 9-fold increases, however, the *TRP*$^+$ reversion frequency appears to be exceptionally low at 8 hours, for both doses of pyrvinium iodide (Table 21). The 6-methyl-analog of pyrvinium iodide definitely induces mutations and the *TRP*$^+$ reversion frequencies increased with increased doses. With the higher doses of the 6-chloro-analog ($2x10^{-4}$ M, $4x10^{-4}$ M, and $8x10^{-4}$ M), the *TRP*$^+$ reversion frequencies were less than the spontaneous levels; at the lower doses ($5x10^{-5}$ M and $1x10^{-4}$ M), the 6-chloro-analog of pyrvinium iodide is only weakly mutagenic (Table 21).

Pyrvinium pamoate, pyrvinium iodide, and the 6-methyl-analog of pyrvinium iodide induced significant increases in the *HOM*$^+$ reversion frequencies; pyrvinium iodide is the most active and pyrvinium pamoate is the least active of these frameshift mutagens (Table 21). The 6-chloro-analog of pyrvinium iodide is a very weak frameshift mutagen.

Pyrvinium iodide induced frameshift mutations at the *his4* and *leu2* loci of strains XY718-1A and 7854-1A of *Saccharomyces cerevisiae* but suppressor events, which were identified by the coreversion analysis of HIS^+ revertants, appear to be induced only in strain 7854-1A. None of the LEU^+ revertants in strain 7854-1A were corevertants (Tables 22 and 23). The 6-methyl-analog of pyrvinium iodide induced significant increases in locus (*his4* and *leu2*) and suppressor events in strains XY718-1A and 7854-1A, and these reversion frequencies increased as the dose was increased. The 6-chloro-analog of pyrvinium iodide induced significant increases in reversions of the *his4-519* and *leu2-3* frameshift mutants, as well as increases in frameshift suppressor events, in strain XY718-1A, however, an effect on strain 7854-1A was not apparent (Tables 22 and 23). Pyrvinium pamoate does not appear to induce frameshift mutations at the *his4* locus in strain XY718-1A, whereas significant increases in HIS^+ reversion frequencies were observed in strain 7854-1A. This dipyrvinium salt appears to have only a weak effect, if any, on the *leu2* locus, and suppressor events were not detected in either strain (Tables 22 and 23).

DISCUSSION

Pyrvinium pamoate does not induce transversions in strain XV185-14C of *Saccharomyces cerevisiae* and pyrvinium iodide induces less than 2-fold increases in locus (*arg4*) and suppressor (ocher) events at 3 hours of exposure. The 6-methyl-analog of pyrvinium iodide induces AT→TA, AT→CG, and GC→TA transversions, whereas the

6-chloro-analog does not induce transversions. Pyrvinium iodide and its 6-methyl-analog induce high levels of transitions, whereas pyrvinium pamoate and the 6-chloro-analog of pyrvinium iodide only induce up to 10-fold increases in *HIS*$^+$ reversion frequencies in strain XV185-14C. The *his1-7* allele can revert by transitions or transversions but the transversion data obtained with the *arg4-17* allele indicates that the pyrvinium salts and the analogs predominantly induce transitions. Pyrvinium iodide and its 6-methyl-analog are active as frameshift mutagens in strains XV185-14C, XY718-1A, and 7854-1A of *Saccharomyces cerevisiae*. The 6-chloro-analog is a very weak frameshift mutagen in strain XV185-14C (*hom3-10* allele) but it is more active than pyrvinium pamoate in strains XY718-1A and 7854-1A. These frameshift mutagens (the pyrvinium salts and the analogs) predominantly induce locus (*his4* and *leu2*) events in strains XY718-1A and 7854-1A.

The induction of frameshift mutations in strain 7854-1A (*his4-38*) by pyrvinium pamoate and the absence of such an effect in strain XY718-1A (*his4-519*) has also been observed with hycanthone (R.C. von Borstel, Z-Q. Chen, and R.C. von Borstel II, unpublished data). The decrease in the mutation frequencies to below the spontaneous levels, which is an effect that occurred at high doses of the 6-chloro-analog of pyrvinium iodide, has also been observed after hycanthone mutagenesis (von Borstel and Igali, 1975). For growing cells, a decrease in the mutation frequency to below the spontaneous level implies that, at high doses of the mutagen, either a mutagenic repair system has become saturated or an accurate repair system has been induced (von Borstel, 1982). For stationary-phase cells, such a decline in the mutation frequency implies that the spontaneous mutants that are already present

are more sensitive to the mutagen than are the nonmutant cells.

Pyrvinium pamoate induces base-substitution mutations (transitions but not transversions), intragenic mitotic recombination, gene conversion, and chromosome breakage that leads to intergenic mitotic recombination. Pyrvinium pamoate is also a frameshift mutagen and frameshift mutations are associated with agents that intercalate into DNA (Lerman, 1961), thus pyrvinium pamoate may also be an intercalating agent. Positively charged, planar chromophores such as the pyrvinium ion, which interact with DNA, are likely to be intercalators. They insert themselves between the base pairs of the nucleic acid polymer. The consequences of intercalation are the lengthening of the double helix (a base-pair separation from 3.38 Å to 6.76 Å implies intercalation) and the unwinding of supercoiled, closed-circular DNA (Miller and Newlin, 1982). With a topoisomerase-fluorescence assay (Lee and Morgan, 1978), it has been determined that pyrvinium pamoate can unwind supercoiled DNA as effectively as can the antimalarial drug chloroquine (P. Dickie and A.R. Morgan, unpublished data). Chloroquine is a quinoline compound that is known to intercalate (Cohen and Yielding, 1965). In addition, electron microscopy, which is a method that can be used to determine whether or not a drug lengthens DNA (Butour *et al.*, 1978), has demonstrated that pyrvinium iodide (at 300 molecules/base pair) lengthens pBR322 DNA by 11% and that pyrvinium pamoate (at 1500 molecules/base pair) lengthens the plasmid DNA by 12% (P. Dickie and D.G. Scraba, unpublished data).

Pyrvinium appears to interact with DNA by at least two distinct forms of binding, and these are external binding and intercalation. External binding, which occurs by ionic interactions and is presumed to

be a weaker form of binding than intercalation, involves an electrostatic attraction between the chloroquine cation, or the pyrvinium cation, and the anionic phosphate groups in the backbone of the DNA (Cohen and Yielding, 1965). The inhibition of this external binding by competing cations such as Mg^{++} is an indication that these negatively charged groups must be accessible before binding can take place (Cohen and Yielding, 1965).

The antiparasitic drug hycanthone also interacts with DNA (Hirschberg *et al.*, 1968). The hydroxylation of the methyl-substituent of the thioxanthene ring system of lucanthone produced hycanthone (Figure 10). Hycanthone has been reported to be a carcinogen in mice that had been infected with the causative organism of schistosomiasis (Haese *et al.*, 1973; Haese and Bueding, 1976). The drug induced frameshift mutations in the bacterium *Salmonella typhimurium* (Hartman *et al.*, 1971), and in the yeast *Saccharomyces cerevisiae* (Meadows *et al.*, 1973), whereas lucanthone was mutagenic in *Salmonella typhimurium* only after metabolic activation by mammalian liver microsomes (Hartman *et al.*, 1975). Lucanthone was not mutagenic in yeast (von Borstel and Igali, 1975), and this implies that the cytochrome P-450 system of yeast is not identical to that of mammalian liver.

The addition of a 6-chloro-substituent to the thioxanthene ring system of hycanthone results in decreased toxicity to mammals (Bueding and Batzinger, 1977). Similarly, in yeast, the 6-chloro-analog of pyrvinium iodide is less toxic than pyrvinium iodide itself. The interaction of lucanthone with calf thymus DNA *in vitro* requires the proximal nitrogen of the side chain on the thioxanthene ring system, since the conversion of this 2° amine to a 3° amine abolishes the

interaction with DNA (Hirschberg *et al.*, 1968). In yeast, derivatives of lucanthone that have a methyl-substituent at the 4-position of the thioxanthene ring system plus a 2° or 3° amine are not mutagenic, whereas derivatives that have a hydroxylated methyl-substituent at the 4-position plus a 2° or 3° amine are mutagenic (von Borstel and Igali, 1975). The hydroxylated methyl-substituent of hycanthone appears to be responsible for the mutagenic activity of the drug in yeast, and this suggests that yeast does not have the hydroxylating enzymes that convert the promutagen lucanthone into the mutagen, which is hycanthone. These enzymes are present in mammalian liver (Hartman *et al.*, 1975).

The evidence that was obtained with hycanthone can be extended to explain the mutagenicity of the 6-methyl-analog of pyrvinium iodide; perhaps the 6-methyl-substituent is hydroxylated, resulting in a mutagen as potent as pyrvinium iodide. It is possible that the yeast strains that were used by Meadows *et al.* (1973), and by von Borstel and Igali (1975), did not have sufficient levels of hydroxylating enzymes, and variations in the cytochrome P-450 content of different yeast strains have been documented (Callen and Philpot, 1977). Also, the pyrvinium moiety may have an additional active site, which could be the cationic site at the methylated ring nitrogen (see Figure 9). This theory is supported by the diminished, but not abolished, genetic activity of the 6-chloro-analog of pyrvinium iodide.

TABLE 18

Induction of mitotic recombination and aberrant colonies in strain D5 of *Saccharomyces cerevisiae* after treatment with pyrvinium pamoate, pyrvinium iodide, and the chloro- and methyl-analogs of pyrvinium iodide

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival (%)	Total Colonies[a]	Colony Phenotype[b]						Mitotic Recombination[c] (%)	Total Aberrant Colonies[d] (%)
				RP	RPW	R	RW	P	PW		
control (2.5% DMSO)	3	100	2255	0	1	1	0	1	0	0.04	0.1
	8	100	1458	0	0	0	0	1	1	<0.07	0.1
	24	100	1398	0	0	0	1	0	1	<0.07	0.1
pyrvinium pamoate $4x10^{-4}$	3	92	1381	8	1	0	3	2	1	0.6	1.1
	8	80	1168	6	5	3	3	3	3	0.94	2.0
	24	69	970	6	5	1	6	1	1	1.1	2.1
pyrvinium iodide $2x10^{-4}$	3	21	1265	13	3	4	4	7	6	1.3	2.9
	8	5	300	4	0	2	1	1	1	1	3
	24	1	611	4	1	4	2	5	3	0.8	3.1
$4x10^{-4}$	3	11	675	4	3	3	3	1	0	1	2.1
	8	0.7	410	2	2	1	2	2	0	1	2
	24	0.3	1840	5	3	13	4	13	3	0.4	2.2

TABLE 18 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival (%)	Total Colonies[a]	Colony Phenotype[b]						Mitotic Recombination[c] (%)	Total Aberrant Colonies[d] (%)
				RP	RPW	R	RW	P	PW		
6-chloro-analog of pyrvinium iodide $5x10^{-5}$	3	100	1504	3	3	0	1	0	4	0.4	0.73
	8	94	1364	5	3	1	3	0	2	0.6	1.0
	24	79	1109	2	2	2	3	0	1	0.4	0.90
$1x10^{-4}$	3	99	1494	4	3	1	2	1	3	0.6	0.94
	8	88	1282	5	7	1	14	4	3	0.94	2.6
	24	22	1229	3	0	3	3	2	2	0.2	1.1
$2x10^{-4}$	3	39	527	3	1	1	1	1	2	0.8	2
	8	23	1329	5	3	1	2	1	3	0.6	1.1
	24	13	741	1	1	2	2	0	1	0.3	0.9
$4x10^{-4}$	3	19	1140	2	0	1	1	2	2	0.2	0.7
	8	12	718	1	3	1	1	1	1	0.6	1
$8x10^{-4}$	3	4.4	1228	0	0	0	0	1	0	<0.08	0.08
	8	4.0	1080	3	1	0	1	1	2	0.4	0.7

TABLE 18 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival (%)	Total Colonies[a]	Colony Phenotype[b]						Mitotic Recombination[c] (%)	Total Aberrant Colonies[d] (%)
				RP	RPW	R	RW	P	PW		
6-methyl-analog of pyrvinium iodide											
$5x10^{-5}$	3	100	1497	12	6	0	5	4	5	1.2	2.1
	8	42	617	2	2	1	1	2	2	0.6	1.6
	24	21	1181	1	1	2	2	2	2	0.2	0.85
$1x10^{-4}$	3	88	1316	13	3	2	4	9	1	1.2	2.4
	8	19	1111	4	4	4	4	2	4	0.7	2.0
	24	2.5	1404	4	3	4	6	2	2	0.5	1.5
$2x10^{-4}$	3	20	1202	6	3	9	3	5	5	0.8	2.6
	8	2	1220	4	3	5	5	3	4	0.6	2.0
	24	0.2	1058	1	1	3	2	3	3	0.2	1.2
$4x10^{-4}$	3	18	1063	11	3	8	1	5	3	1.3	2.9
	8	2	1093	7	4	1	3	5	2	1.0	2.0
$8x10^{-4}$	3	1.5	898	6	3	6	2	6	4	1	3.0
	8	0.2	1113	6	5	2	4	3	4	0.99	2.2

[a] The total number of surviving colonies on a complete medium containing a limiting concentration of adenine (MC with 5 µg adenine/ml).

[b] The abbreviations RP, RPW, R, RW, P, and PW refer to red-pink, red-pink-white, red, red-white, pink, and pink-white colonies, respectively.

[c] Represented by RP (twin-spot) and RPW colonies.

[d] Includes RP, RPW, R, RW, P, and PW colonies.

TABLE 19

Induction of intragenic mitotic recombination, intergenic mitotic recombination, and aberrant colonies in strain D7 of *Saccharomyces cerevisiae* after treatment with pyrvinium pamoate, pyrvinium iodide, and the chloro- and methyl-analogs of pyrvinium iodide

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[a] (%)	Allelic Recombination Frequencies[b] (TRP^+ prototrophs /10^5 survivors)	Colony Phenotype[c]						Mitotic Recombination[d] (%)	Total Aberrant Colonies[e] (%)
				RP	RPW	R	RW	P	PW		
control (2.5% DMSO)	3	100 (1360)	1 (404)	0	0	0	1	2	0	<0.07	0.2
	8	100 (1288)	1 (448)	0	0	0	0	1	1	<0.08	0.2
	24	100 (1216)	1 (197)	0	1	0	0	0	1	0.08	0.2
pyrvinium pamoate $4x10^{-4}$	3	88 (1200)	2 (617)	2	0	0	0	0	2	0.2	0.3
	8	65 (836)	3 (679)	14	1	1	1	1	1	1.8	2.7
	24	54 (656)	4 (580)	5	4	4	1	0	0	1.4	2.1
pyrvinium iodide $2x10^{-4}$	3	10.2 (3062)	9 (301)	11	3	4	4	8	1	0.46	1.0
	8	2.5 (1287)	163 (1259)	24	4	17	6	13	6	2.2	5.4
	24	0.9 (2453)	198 (529)	27	4	21	6	28	6	1.3	3.8
$4x10^{-4}$	3	9.7 (526)	12 (374)	12	0	6	2	3	1	2.3	4.6
	8	0.1 (487)	4672 (1365)	5	3	12	4	16	1	2	8.4
	24	0.02(3665)	<20 (0)	29	6	12	6	6	2	0.96	1.7

TABLE 19 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[a] (%)		Allelic Recombination Frequencies[b] (*TRP*+ prototrophs /10^5 survivors)		Colony Phenotype[c]						Mitotic Recombination[d] (%)	Total Aberrant Colonies[e] (%)
						RP	RPW	R	RW	P	PW		
6-chloro-analog of pyrvinium iodide $5x10^{-5}$	3	93	(1266)	2	(468)	5	2	0	1	1	2	0.6	0.87
	8	82	(1050)	3	(689)	5	3	3	0	0	0	0.8	1.0
	24	36	(437)	7	(732)	1	0	1	2	4	0	0.2	2
$1x10^{-4}$	3	86	(1176)	2	(544)	6	2	3	0	6	2	0.7	1.6
	8	80	(1024)	4	(1002)	16	3	3	0	2	0	1.9	2.3
	24	27	(832)	13	(1067)	7	1	3	1	2	3	1	2.0
$2x10^{-4}$	3	84	(1139)	1	(383)	1	0	1	0	0	2	0.09	0.4
	8	75	(3876)	4	(876)	19	4	3	5	2	1	0.59	0.88
	24	9	(424)	23	(582)	1	6	2	0	2	0	2	2.6
$4x10^{-4}$	3	83	(1123)	0.02	(6)	1	0	0	0	0	0	0.09	0.09
	8	46	(2382)	0.03	(4)	1	1	0	2	0	1	0.08	0.2
	24	3	(1464)	0.3	(3)	1	1	2	0	3	0	0.1	0.5
$8x10^{-4}$	3	77	(1051)	0.01	(3)	0	0	0	0	0	0	<0.1	<0.1
	8	33	(1696)	0.02	(2)	2	0	0	0	0	1	0.1	0.2
	24	3	(1425)	<0.1	(0)	1	0	0	0	2	0	0.07	0.2

TABLE 19 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[a] (%)		Allelic Recombination Frequencies[b] (*TRP*+ prototrophs /10^5 survivors)		Colony Phenotype[c]						Mitotic Recombination[d] (%)	Total Aberrant Colonies[e] (%)
						RP	RPW	R	RW	P	PW		
6-methyl-analog of pyrvinium iodide													
$5x10^{-5}$	3	85	(1160)	2	(590)	7	3	3	0	8	2	0.86	2.0
	8	43	(1394)	15	(2052)	26	2	8	1	20	1	2.0	4.2
	24	2	(847)	131	(667)	3	3	5	2	2	0	0.7	1.8
$1x10^{-4}$	3	82	(1115)	6	(1512)	15	5	2	2	3	0	1.8	2.4
	8	8	(431)	132	(3400)	9	4	8	3	10	0	3.0	7.9
	24	0.1	(278)	100	(17)	1	1	1	1	2	0	0.7	2.2
$2x10^{-4}$	3	77	(1053)	9	(2352)	10	5	3	4	6	3	1.4	2.9
	8	8	(422)	133	(3378)	7	5	6	3	10	2	2.8	7.8
	24	0.004	(1721)	500	(7)	36	10	6	6	7	0	2.7	3.8
$4x10^{-4}$	3	76	(4109)	9	(2251)	34	5	5	3	4	3	0.95	1.3
	8	5	(1377)	158	(2374)	31	5	19	3	44	6	2.6	7.84
$8x10^{-4}$	3	75	(4070)	6	(1578)	25	6	4	1	9	7	0.76	1.3
	8	0.3	(1542)	1984	(1836)	36	9	13	7	16	0	2.9	5.2

TABLE 19 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[a] (%)	Allelic Recombination Frequencies[b] (*TRP*+ prototrophs /10^5 survivors)	Colony Phenotype[c]						Mitotic Recombination[d] (%)	Total Aberrant Colonies[e] (%)
				RP	RPW	R	RW	P	PW		

[a]The figures in parentheses constitute the total number of surviving colonies on a complete medium containing a limiting concentration of adenine (MC with 5 µg adenine/ml plus isoleucine and valine).

[b]The figures in parentheses constitute the total number of prototrophic colonies on 4 plates of omission medium (MC minus tryptophan plus isoleucine and valine).

[c]The abbreviations RP, RPW, R, RW, P, and PW refer to red-pink, red-pink-white, red, red-white, pink, and pink-white colonies, respectively.

[d]Represented by RP (twin-spot) and RPW colonies.

[e]Includes RP, RPW, R, RW, P, and PW colonies.

TABLE 20

Reversion frequencies (*ARG*$^+$) induced in strain XV185-14C of *Saccharomyces cerevisiae* after treatment with pyrvinium pamoate, pyrvinium iodide, and the chloro- and methyl-analogs of pyrvinium iodide

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)		
			Locus	*ARG*$^+$ Suppressors	Total
control (2.5% water)	3	100 (936)	1 (13)	28 (324)	29 (337)
	8	100 (921)	1 (12)	30 (346)	31 (358)
	24	100 (755)	1 (11)	40 (380)	41 (391)
control (2.5% DMSO)	3	94 (879)	1 (11)	37 (400)	38 (411)
	8	99 (909)	2 (18)	42 (479)	44 (497)
	24	88 (661)	1 (10)	46 (382)	47 (392)
pyrvinium pamoate $4x10^{-4}$	3	92 (861)	0.2 (2)	34 (363)	34 (365)
	8	87 (802)	0.5 (5)	42 (424)	43 (429)
	24	62 (469)	0.7 (4)	65 (382)	66 (386)
pyrvinium iodide $2x10^{-4}$	3	63 (589)	5 (36)	43 (315)	48 (351)
	8	42 (391)	1 (3)	92 (448)	93 (451)
	24	7 (211)	<2 (0)	110 (69)	110 (69)
$4x10^{-4}$	3	31 (1174)	4 (15)	65 (238)	69 (253)
	8	19 (701)	<0.5 (0)	93 (204)	93 (204)
	24	1 (26)	<12 (0)	90 (7)	90 (7)

TABLE 20 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)		
			Locus	*ARG*+ Suppressors	Total
6-chloro-analog of pyrvinium iodide $5x10^{-5}$	3	97 (912)	0.2 (2)	35 (395)	35 (397)
	8	91 (841)	0.4 (4)	42 (437)	42 (441)
	24	56 (419)	0.4 (2)	52 (272)	52 (274)
$1x10^{-4}$	3	96 (897)	0.5 (5)	41 (460)	42 (465)
	8	85 (786)	0.6 (6)	45 (440)	46 (446)
	24	30 (227)	0.4 (1)	61 (172)	61 (173)
$2x10^{-4}$	3	71 (663)	1 (10)	49 (408)	50 (418)
	8	70 (648)	1 (7)	44 (352)	45 (359)
	24	20 (589)	<0.5 (0)	71 (131)	71 (131)
$4x10^{-4}$	3	56 (526)	<0.2 (0)	21 (137)	21 (137)
	8	23 (859)	<0.6 (0)	35 (94)	35 (94)
	24	17 (503)	<0.6 (0)	47 (74)	47 (74)
$8x10^{-4}$	3	29 (1072)	<0.3 (0)	17 (56)	17 (56)
	8	10 (385)	<0.8 (0)	27 (33)	27 (33)
	24	8 (230)	<1 (0)	43 (31)	43 (31)

TABLE 20 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)		
			Locus	*ARG*+ Suppressors	Total
6-methyl-analog of pyrvinium iodide					
$5x10^{-5}$	3	90 (842)	1 (8)	43 (452)	44 (460)
	8	82 (752)	1 (9)	52 (489)	53 (498)
	24	25 (742)	2 (4)	66 (153)	68 (157)
$1x10^{-4}$	3	85 (799)	0.5 (5)	45 (450)	46 (455)
	8	74 (678)	0.6 (5)	58 (490)	59 (495)
	24	2 (546)	<6 (0)	40 (6)	40 (6)
$2x10^{-4}$	3	78 (733)	2 (17)	39 (358)	41 (375)
	8	26 (954)	2 (5)	105 (312)	107 (317)
	24	0.01(35)	<1000 (0)	2000 (2)	2000 (2)
$4x10^{-4}$	3	71 (665)	1 (8)	54 (447)	55 (455)
	8	7 (248)	5 (4)	337 (261)	342 (265)
$8x10^{-4}$	3	31 (1156)	2 (7)	106 (384)	108 (391)
	8	4 (1367)	7 (3)	258 (110)	265 (113)

[a]The figures in parentheses constitute the total number of revertant colonies, as determined by plate counts. Pink revertants were scored as locus events and white revertants were scored as suppressor events. Wherever revertant counts of zero appear, reversion frequencies of less than the value corresponding to one revertant colony are reported.

[b]The figures in parentheses constitute the total number of surviving colonies on 4 plates of complete medium (2 plates of MC and 2 plates of YEPD).

TABLE 21

Reversion frequencies (*HIS*$^+$, *TRP*$^+$, and *HOM*$^+$) induced in strain XV185-14C of *Saccharomyces cerevisiae* after treatment with pyrvinium pamoate, pyrvinium iodide, and the chloro- and methyl-analogs of pyrvinium iodide

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)		
			HIS$^+$	*TRP*$^+$	*HOM*$^+$
control (2.5% DMSO)	3	100 (614)	4 (9)	8 (20)	3 (8)
	8	100 (631)	4 (9)	7 (17)	<1 (0)
	24	100 (630)	3 (8)	6 (15)	2 (8)
pyrvinium pamoate $4x10^{-4}$	3	79 (483)	8 (15)	9 (17)	5 (9)
	8	74 (469)	8 (15)	12 (22)	3 (12)
	24	67 (422)	28 (48)	9 (16)	17 (59)
pyrvinium iodide $2x10^{-4}$	3	40 (243)	397 (386)	65 (63)	18 (36)
	8	45 (709)	257 (292)	8 (9)	20 (45)
	24	6 (1469)	270 (40)	50 (8)	20 (7)
$4x10^{-4}$	3	18 (448)	38 (17)	22 (10)	3 (3)
	8	14 (353)	160 (55)	<1 (0)	3 (2)
	24	5 (1125)	210 (24)	30 (3)	30 (7)

TABLE 21 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)		
			HIS$^+$	*TRP*$^+$	*HOM*$^+$
6-chloro-analog of pyrvinium iodide					
$5x10^{-5}$	3	84 (519)	5 (10)	11 (22)	1 (5)
	8	79 (499)	7 (14)	8 (16)	3 (11)
	24	62 (980)	18 (29)	16 (25)	4 (12)
$1x10^{-4}$	3	78 (479)	14 (27)	21 (41)	2 (7)
	8	71 (450)	10 (18)	8 (15)	2 (9)
	24	27 (431)	30 (21)	<1 (0)	6 (9)
$2x10^{-4}$	3	76 (466)	22 (42)	4 (7)	5 (9)
	8	60 (381)	16 (25)	3 (5)	3 (8)
	24	11 (216)	60 (16)	<4 (0)	9 (5)
$4x10^{-4}$	3	75 (460)	4 (7)	1 (1)	1 (3)
	8	16 (100)	20 (6)	10 (5)	2 (2)
$8x10^{-4}$	3	74 (1816)	<1 (0)	<1 (0)	<1 (0)
	8	7 (1800)	6 (1)	<6 (0)	<3 (0)

TABLE 21 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^7 survivors)		
			HIS$^+$	*TRP*$^+$	*HOM*$^+$
6-methyl-analog of pyrvinium iodide					
5×10^{-5}	3	83 (508)	9 (18)	12 (24)	3 (12)
	8	84 (530)	15 (32)	13 (28)	2 (10)
	24	14 (218)	92 (32)	10 (5)	10 (9)
1×10^{-4}	3	72 (442)	35 (62)	12 (22)	4 (15)
	8	73 (462)	17 (32)	13 (24)	5 (17)
	24	1 (30)	<30 (0)	<30 (0)	<20 (0)
2×10^{-4}	3	63 (389)	84 (130)	56 (87)	6 (19)
	8	24 (371)	61 (36)	18 (11)	7 (8)
	24	0.03 (7)	<1000 (0)	<1000 (0)	<700 (0)
4×10^{-4}	3	58 (353)	179 (253)	49 (69)	14 (39)
	8	9 (216)	310 (66)	46 (10)	20 (8)
8×10^{-4}	3	56 (1365)	88 (120)	<1 (0)	11 (29)
	8	8 (192)	160 (30)	<5 (0)	20 (7)

[a]The figures in parentheses constitute the total number of revertant colonies, as determined by plate counts. Wherever revertant counts of zero appear, reversion frequencies of less than the value corresponding to one revertant colony are reported.

[b]The figures in parentheses constitute the total number of surviving colonies on 4 plates of MC medium.

TABLE 22

Reversion frequencies induced in strain XY718-1A (a *his4-519 leu2-3*) of *Saccharomyces cerevisiae* after treatment with pyrvinium pamoate, pyrvinium iodide, and the chloro- and methyl-analogs of pyrvinium iodide

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^8 survivors)					
			HIS$^+$			*LEU*$^+$		
			Locus	Suppressors	Total	Locus	Suppressors	Total
control (2.5% DMSO)	3	100 (463)	<2 (0)	<2 (0)	<2 (0)	<2 (0)	<2 (0)	<2 (0)
	8	100 (423)	<2 (0)	<2 (0)	<2 (0)	5 (2)	<2 (0)	5 (2)
	24	100 (420)	<2 (0)	<2 (0)	<2 (0)	5 (2)	<2 (0)	5 (2)
pyrvinium pamoate $4x10^{-4}$	3	97 (448)	<2 (0)	<2 (0)	<2 (0)	<2 (0)	<2 (0)	<2 (0)
	8	76 (320)	<3 (0)	<3 (0)	<3 (0)	6 (2)	<3 (0)	6 (2)
	24	48 (202)	<5 (0)	<5 (0)	<5 (0)	5 (1)	<5 (0)	5 (1)
pyrvinium iodide $2x10^{-4}$	3	17 (79)	10 (1)	<10 (0)	10 (1)	40 (3)	<10 (0)	40 (3)
	8	6 (102)	80 (2)	<40 (0)	80 (2)	100 (3)	<40 (0)	100 (3)
	24	1 (164)	<5 (0)	<5 (0)	<5 (0)	<5 (0)	<5 (0)	<5 (0)
$4x10^{-4}$	3	8 (38)	<30 (0)	<30 (0)	<30 (0)	<30 (0)	<30 (0)	<30 (0)
	8	4 (68)	60 (1)	<60 (0)	60 (1)	<60 (0)	<60 (0)	<60 (0)
	24	0.4(67)	<600 (0)	<600 (0)	<600 (0)	<600 (0)	<600 (0)	<600 (0)

TABLE 22 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^8 survivors)					
			HIS$^+$			*LEU*$^+$		
			Locus	Suppressors	Total	Locus	Suppressors	Total
6-chloro-analog of pyrvinium iodide 5×10^{-5}	3	98 (453)	<2 (0)	<2 (0)	<2 (0)	<2 (0)	<2 (0)	<2 (0)
	8	96 (408)	<2 (0)	<2 (0)	<2 (0)	2 (1)	<2 (0)	2 (1)
	24	41 (174)	<6 (0)	<6 (0)	<6 (0)	<6 (0)	<6 (0)	<6 (0)
1×10^{-4}	3	96 (446)	2 (1)	<2 (0)	2 (1)	4 (2)	<2 (0)	4 (2)
	8	96 (407)	<2 (0)	<2 (0)	<2 (0)	5 (2)	<2 (0)	5 (2)
	24	39 (661)	6 (1)	<6 (0)	6 (1)	<6 (0)	<6 (0)	<6 (0)
2×10^{-4}	3	78 (362)	3 (1)	3 (1)	6 (2)	6 (2)	3 (1)	9 (3)
	8	66 (280)	<4 (0)	<4 (0)	<4 (0)	7 (2)	4 (1)	10 (3)
	24	31 (518)	<8 (0)	<8 (0)	<8 (0)	<8 (0)	<8 (0)	<8 (0)
4×10^{-4}	3	60 (227)	4 (1)	4 (1)	7 (2)	7 (2)	<4 (0)	7 (2)
	8	57 (959)	<4 (0)	<4 (0)	<4 (0)	12 (3)	<4 (0)	12 (3)
	24	17 (355)	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)
8×10^{-4}	3	59(1091)	4 (1)	<4 (0)	4 (1)	7 (2)	<4 (0)	7 (2)
	8	50 (847)	<5 (0)	<5 (0)	<5 (0)	5 (1)	<5 (0)	5 (1)
	24	2 (36)	<100 (0)	<100 (0)	<100 (0)	<100 (0)	<100 (0)	<100 (0)

TABLE 22 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^8 survivors)					
			HIS$^+$			*LEU*$^+$		
			Locus	Suppressors	Total	Locus	Suppressors	Total
6-methyl-analog of pyrvinium iodide								
5×10^{-5}	3	89 (410)	<2 (0)	<2 (0)	<2 (0)	10 (5)	2 (1)	10 (6)
	8	88 (374)	3 (1)	<3 (0)	3 (1)	3 (1)	<3 (0)	3 (1)
	24	18 (298)	10 (1)	<10 (0)	10 (1)	30 (2)	<10 (0)	30 (2)
1×10^{-4}	3	83 (384)	8 (3)	<3 (0)	8 (3)	20 (9)	<3 (0)	20 (9)
	8	77 (325)	3 (1)	3 (1)	6 (2)	6 (2)	<3 (0)	6 (2)
	24	1 (177)	<200 (0)	<200 (0)	<200 (0)	<200 (0)	<200 (0)	<200 (0)
2×10^{-4}	3	74 (342)	3 (1)	3 (1)	6 (2)	30 (9)	<3 (0)	30 (9)
	8	71 (302)	3 (1)	3 (1)	7 (2)	<3 (0)	<3 (0)	10 (3)
	24	0.003(5)	<8000 (0)	<8000 (0)	<8000 (0)	<8000 (0)	<8000 (0)	<8000 (0)
4×10^{-4}	3	50 (229)	4 (1)	<4 (0)	4 (1)	9 (2)	20 (4)	30 (6)
	8	11 (186)	<20 (0)	20 (1)	20 (1)	20 (1)	<20 (0)	20 (1)
8×10^{-4}	3	9 (170)	<20 (0)	<20 (0)	<20 (0)	20 (1)	<20 (0)	20 (1)
	8	3 (44)	<90 (0)	<90 (0)	<90 (0)	<90 (0)	<90 (0)	<90 (0)

[a] The figures in parentheses constitute the total number of revertant colonies, as determined by plate counts. Suppressor events were identified by corevertants (His^+Leu^+ phenotype). Wherever revertant counts of zero appear, reversion frequencies of less than the value corresponding to one revertant colony are reported.

[b] The figures in parentheses constitute the total number of surviving colonies on 4 plates of YEPD-agar.

TABLE 23

Reversion frequencies induced in strain 7854-1A (a *his4-38 leu2-3*) of *Saccharomyces cerevisiae* after treatment with pyrvinium pamoate, pyrvinium iodide, and the chloro- and methyl-analogs of pyrvinium iodide

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^8 survivors)					
			HIS$^+$			*LEU*$^+$		
			Locus	Suppressors	Total	Locus	Suppressors	Total
control (2.5% DMSO)	3	100 (497)	<2 (0)	<2 (0)	<2 (0)	2 (1)	<2 (0)	2 (1)
	8	100 (440)	<2 (0)	<2 (0)	<2 (0)	2 (1)	<2 (0)	2 (1)
	24	100 (423)	<2 (0)	2 (1)	2 (1)	5 (2)	<2 (0)	5 (2)
pyrvinium pamoate $4x10^{-4}$	3	92 (459)	<2 (0)	<2 (0)	<2 (0)	<2 (0)	<2 (0)	<2 (0)
	8	79 (349)	10 (4)	<3 (0)	10 (4)	6 (2)	<3 (0)	6 (2)
	24	48 (202)	5 (1)	<5 (0)	5 (1)	5 (1)	<5 (0)	5 (1)
pyrvinium iodide $2x10^{-4}$	3	8 (161)	20 (1)	20 (1)	50 (2)	20 (1)	<20 (0)	20 (1)
	8	4 (79)	<50 (0)	<50 (0)	<50 (0)	50 (1)	<50 (0)	50 (1)
	24	0.1(112)	<400 (0)	<400 (0)	<400 (0)	<400 (0)	<400 (0)	<400 (0)
$4x10^{-4}$	3	4 (77)	50 (1)	50 (1)	100 (2)	50 (1)	<50 (0)	50 (1)
	8	0.1 (17)	<200 (0)	<200 (0)	<200 (0)	<200 (0)	<200 (0)	<200 (0)
	24	0.01(10)	<4000 (0)	<4000 (0)	<4000 (0)	<4000 (0)	<4000 (0)	<4000 (0)

TABLE 23 (continued)

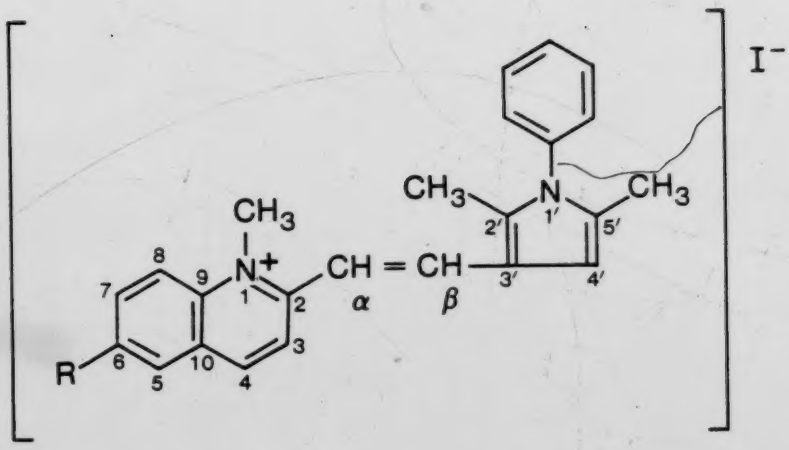
Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^8 survivors)					
			HIS$^+$			*LEU*$^+$		
			Locus	Suppressors	Total	Locus	Suppressors	Total
6-chloro-analog of pyrvinium iodide $5x10^{-5}$	3	89 (444)	<2 (0)	<2 (0)	<2 (0)	<2 (0)	<2 (0)	<2 (0)
	8	89 (392)	<3 (0)	<3 (0)	<3 (0)	3 (1)	<3 (0)	3 (1)
	24	59 (251)	4 (1)	<4 (0)	4 (1)	<4 (0)	<4 (0)	<4 (0)
$1x10^{-4}$	3	86 (428)	<2 (0)	<2 (0)	<2 (0)	2 (1)	<2 (0)	2 (1)
	8	74 (326)	<3 (0)	3 (1)	3 (1)	3 (1)	<3 (0)	3 (1)
	24	22 (374)	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)
$2x10^{-4}$	3	63 (314)	3 (1)	<3 (0)	3 (1)	<3 (0)	3 (1)	3 (1)
	8	50 (218)	<5 (0)	<5 (0)	<5 (0)	5 (1)	5 (1)	10 (2)
	24	21 (352)	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)
$4x10^{-4}$	3	28 (140)	<7 (0)	<7 (0)	<7 (0)	7 (1)	<7 (0)	7 (1)
	8	24 (105)	<10 (0)	<10 (0)	<10 (0)	10 (1)	<10 (0)	10 (1)
	24	10 (166)	<20 (0)	<20 (0)	<20 (0)	<20 (0)	<20 (0)	<20 (0)
$8x10^{-4}$	3	27 (545)	<7 (0)	<7 (0)	<7 (0)	7 (1)	<7 (0)	7 (1)
	8	23 (403)	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)	<10 (0)
	24	9 (153)	<30 (0)	<30 (0)	<30 (0)	<30 (0)	<30 (0)	<30 (0)

TABLE 23 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival[b] (%)	Reversion Frequencies[a] (revertants/10^8 survivors)					
			HIS$^+$			*LEU*$^+$		
			Locus	Suppressors	Total	Locus	Suppressors	Total
6-methyl-analog of pyrvinium iodide								
$5x10^{-5}$	3	82 (409)	5 (2)	<2 (0)	5 (2)	5 (2)	2 (1)	7 (3)
	8	52 (230)	9 (2)	<4 (0)	9 (2)	9 (2)	<4 (0)	9 (2)
	24	22 (371)	<10 (0)	<10 (0)	<10 (0)	10 (1)	<10 (0)	10 (1)
$1x10^{-4}$	3	53 (264)	8 (2)	<4 (0)	8 (2)	20 (4)	<4 (0)	20 (4)
	8	21 (92)	40 (4)	<10 (0)	40 (4)	30 (3)	<10 (0)	30 (3)
	24	1 (203)	<200 (0)	<200 (0)	<200 (0)	<200 (0)	<200 (0)	<200 (0)
$2x10^{-4}$	3	50 (250)	4 (1)	4 (1)	8 (2)	20 (5)	<4 (0)	20 (5)
	8	14 (62)	<20 (0)	<20 (0)	<20 (0)	20 (1)	<20 (0)	20 (1)
	24	0.02(35)	<1000 (0)	<1000 (0)	<1000 (0)	<1000 (0)	<1000 (0)	<1000 (0)
$4x10^{-4}$	3	48 (238)	<4 (0)	4 (1)	4 (1)	4 (1)	8 (2)	10 (3)
	8	4 (68)	<60 (0)	<60 (0)	<60 (0)	<60 (0)	<60 (0)	<60 (0)
$8x10^{-4}$	3	27 (545)	7 (1)	<7 (0)	7 (1)	7 (1)	<7 (0)	7 (1)
	8	1 (20)	<200 (0)	<200 (0)	<200 (0)	<200 (0)	<200 (0)	<200 (0)

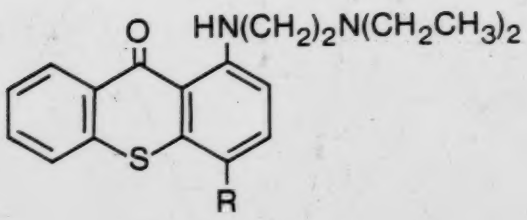
[a]The figures in parentheses constitute the total number of revertant colonies, as determined by plate counts. Suppressor events were identified by corevertants (His^+Leu^+ phenotype). Wherever revertant counts of zero appear, reversion frequencies of less than the value corresponding to one revertant colony are reported.

[b]The figures in parentheses constitute the total number of surviving colonies on 4 plates of YEPD-agar.

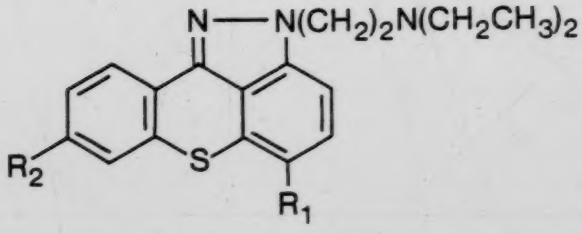


	R	MW
pyrvinium iodide	$-N(CH_3)_2$	509.45
6-chloro-analog	$-Cl$	500.83
6-methyl-analog	$-CH_3$	480.41

FIGURE 9. A summary of the structural modifications and the molecular weights of the 6-chloro- and 6-methyl-analogs of 6-dimethylamino-2-[2-(2',5'-dimethyl-1'-phenyl-3'-pyrrolyl)vinyl]-1-methylquinolinium iodide, which is referred to as pyrvinium iodide.



	R	MW
lucanthone	$-CH_3$	340.48
hycanthone	$-CH_2OH$	356.48



	R_1	R_2	MW
IA-3	$-CH_3$	$-Cl$	371.92
IA-4	$-CH_2OH$	$-Cl$	387.92
IA-5	$-CH_3$	$-H$	337.48
IA-6	$-CH_2OH$	$-H$	353.48

FIGURE 10. A summary of the structural formulas and molecular weights of the antischistosomal drugs lucanthone, hycanthone, IA-3 (6-chloroindazole analog of lucanthone), IA-4 (6-chloroindazole analog of hycanthone), IA-5 (indazole analog of lucanthone), and IA-6 (indazole analog of hycanthone).

ARTICLE V

The Induction of Cytoplasmic, Respiratory-Deficient Variants in *Saccharomyces cerevisiae* with Pyrvinium Salts, Analogs of Pyrvinium, and Ethidium Bromide

ABSTRACT

Cytoplasmic, respiratory-deficient variants (petites) were induced in the yeast *Saccharomyces cerevisiae* with pyrvinium pamoate, pyrvinium iodide, and the 6-chloro- and 6-methyl-analogs of pyrvinium iodide, as well as with ethidium bromide. Pyrvinium pamoate induced petites less readily than did ethidium bromide, however, both ethidium bromide and the dipyrvinium salt (pyrvinium pamoate) were more efficient than the monopyrvinium salt (pyrvinium iodide) and its analogs at inducing petites. The primary mechanism of action of ethidium bromide and pyrvinium pamoate, which are anthelmintic drugs, may be the destruction of the mitochondrial DNA of helminths.

INTRODUCTION

During the testing of pyrvinium pamoate for potential genetic activity in growing cells of strain D5 of *Saccharomyces cerevisiae*, it was observed that the drug induces cytoplasmic, respiratory-deficient variants (von Borstel *et al.*, 1981b). These yeast cells, which are referred to as petites, have lost the respiratory function in their mitochondria, and this may be due to a partial or a total loss of mitochondrial genetic material. The loss of oxidative respiration results in the slowed growth of these yeast cells on glucose-containing media, thus the colonies are smaller in size than those of respiratory-competent cells. An additional method of discerning between petite and grande colonies is by noting the color of the colonies approximately

5 days after plating; the grande colonies are ivory in color, whereas the petite colonies are white.

Although the cells had been cultured in a glucose-containing medium, which permits the growth of respiratory-deficient variants, the control frequency of petites was only 1% (von Borstel *et al.*, 1981b). This indicates that the D5 strain of *Saccharomyces cerevisiae* generates spontaneous petites at low frequencies. Pyrvinium pamoate appeared to induce petites at frequencies approaching 100%, and this was observed with each of the doses that had been used (4.25×10^{-6} M, 4.25×10^{-5} M, and 4.25×10^{-4} M), after 3 hours of treatment (von Borstel *et al.*, 1981b).

It has been suggested that the mitochondrial genetic system is the primary target of chemical carcinogens (cf. Wilkie and Evans, 1982), and the high frequency of petites induced by pyrvinium pamoate indicated that this action of the drug should be studied further.

MATERIALS AND METHODS

Yeast Strains

Strains D5 (genotype $\frac{\alpha}{a}$ $\frac{trp1\text{-}1}{+}$ $\frac{ade2\text{-}40}{ade2\text{-}119}$ $\frac{MAL1}{+}$ $\frac{+}{MAL4}$) and N123 (genotype a *his1*) of *Saccharomyces cerevisiae* were grown on YEPG-agar (1.0% yeast extract, 2.0% peptone, 2.0% glycerol, and 1.5% agar) for 48 hours at 30°C. Cell cultures were prepared in liquid YEPG medium (1% yeast extract, 2% peptone, and 4% glycerol) and incubated for 16 hours in a waterbath shaker at 30°C. The cells were harvested by centrifugation, washed once with 0.1 M sodium phosphate buffer (pH 7.0), resuspended in

liquid YEPD medium (contains 2% dextrose as the carbon source), and these cell suspensions were adjusted to $1x10^7$ cells/ml with liquid YEPD.

Drugs and Solutions

Pyrvinium pamoate (Parke-Davis Canada Incorporated) was used to induce petites in the diploid strain D5 of yeast. The stock solution (184.0 mg/ml) and the dilutions were prepared in dimethylsulfoxide (DMSO).

Pyrvinium pamoate, pyrvinium iodide, and the 6-chloro- and 6-methyl-analogs of pyrvinium iodide, which had been custom-synthesized by Lancaster Synthesis Limited (Eastgate, Lancashire, England), were used to induce petites in the haploid strain N123 of yeast. Stock solutions of pyrvinium pamoate (18.4 mg/ml), pyrvinium iodide (16.3 mg/ml), the 6-chloro-analog (4.0 mg/ml), and the 6-methyl-analog (3.8 mg/ml) were prepared in, and diluted with, DMSO.

The stock solution of ethidium bromide (Sigma Chemical Company), the positive control that was used to induce petites in strains D5 and N123 of *Saccharomyces cerevisiae*, was prepared in sterile water (0.4 mg/ml).

Induction of Petites in Growing Cells

The adjusted cell suspensions ($1x10^7$ cells/ml liquid YEPD) of strains D5 and N123 were added to the control and treatment tubes (3.9 ml/tube). In addition, the control tubes received 0.1 ml of sterile water or 0.1 ml of DMSO, and the treatment tubes received 0.1 ml of a stock solution, or a dilution of a stock solution, of a drug. The tubes were incubated at 30°C in a waterbath shaker; cell samples were taken at

the appropriate time intervals and diluted with phosphate buffer. These dilutions were spread (0.2 ml/plate) onto regular YEPD-agar (contains 2% dextrose) and onto fortified YEPD-agar (contains 10% dextrose). The plates were incubated at 30°C for 3-4 days and the proportion of petite colonies was determined by the tetrazolium-overlay method (Ogur *et al.*, 1957). The tetrazolium dye (2,3,5-triphenyltetrazolium chloride) was used to distinguish the petite colonies from the grande colonies. Grande colonies oxidize the dye to a red oxidation product, whereas petite colonies are incapable of this oxidation. The adenine-dependent colonies of strain D5 (colonies with red-pink, red-pink-white, red, red-white, pink, and pink-white phenotypes), which could not be scored as petite or grande by the overlay method, were patched onto YEPD-agar and replica-plated onto YEPG-agar (Table 24). The colonies with a white phenotype were then analyzed by the tetrazolium-overlay technique (Table 25).

RESULTS AND DISCUSSION

A petite variant of yeast is characterized by defective mitochondrial DNA and the inability to grow on nonfermentable substrates such as glycerol. Generally, the cytoplasmic petite strains of *Saccharomyces cerevisiae* are classified as rho$^-$ (partial deletion of mitochondrial DNA) and rho° (total deletion of mitochondrial DNA). The deletion, or partial deletion, of the DNA of the mitochondria makes it impossible for the cells to utilize glycerol as a carbon source because certain elements of the electron-transport chain (cytochrome *c*

oxidase, apocytochrome *b*, and spectrum cytochromes [*a* and a_3]) are encoded by the mitochondrial DNA, and these elements are absent or defective in petite strains of yeast (Dujon, 1981). The glycerol phosphate shuttle is inoperative without an intact mitochondrial respiratory assembly (Lehninger, 1970). The yeast strains D5 and N123 were grown in a glycerol-containing medium to remove all petite variants before any of the mutagens were added. Ethidium bromide induces respiratory-deficient variants in yeast (Slonimski *et al.*, 1968), therefore it served as a positive control in examining the petites that were induced by pyrvinium pamoate.

It is evident that pyrvinium pamoate induces petites less readily than does ethidium bromide (Tables 25 and 26). In strain D5, a 2-hour exposure to pyrvinium pamoate was required for petite induction, whereas all of the cells became respiratory-deficient after 30 minutes of exposure to ethidium bromide. In strain N123, at least 4 hours of exposure to pyrvinium pamoate were required for petite induction, and more than 50% of the cells had become respiratory-deficient after 8 hours of exposure to the drug (Table 26). Even ethidium bromide, which was used at the same dose (2.5×10^{-5} M) with each yeast strain, did not induce as high a frequency of petites in strain N123 as it did in strain D5. Also, the spontaneous level of petite production in strain N123 was much higher than in strain D5, as is evident from the data for distilled water and DMSO (Tables 25 and 26).

The spontaneous and induced levels of petites in strain D5 (Table 25) are lower than the levels that have been reported previously (von Borstel *et al.*, 1981b), although similar concentrations of pyrvinium pamoate (in the range of 4×10^{-9} M - 4×10^{-3} M) were used in both

experiments. These differences can be attributed to the culture conditions that were used, and to the technique that was used to distinguish between the petite and grande colonies. For example, von Borstel *et al.* (1981b) did not use a glycerol-containing medium to remove the spontaneous petites, and the color difference between the petite (white) and grande (ivory) colonies is an inadequate method of classification because the proportion of petite colonies may be overestimated. The tetrazolium-overlay technique is a more stringent assay for the identification of petite and grande colonies.

It was of especial interest to note that most of the petite colonies that were induced by pyrvinium pamoate turned light red or pink after 24 hours of exposure to the tetrazolium dye, whereas those arising from the cells treated with ethidium bromide remained white in color. The grande colonies turned deep red immediately upon exposure to the tetrazolium dye. This implies that the petites induced by pyrvinium pamoate resulted from a partial loss of mitochondrial DNA, whereas ethidium bromide causes a total loss of mitochondrial DNA (rho° phenotype).

Pyrvinium pamoate is more efficient at inducing petites than are either pyrvinium iodide or the analogs of pyrvinium iodide, which produce petite frequencies of less than 20% in strain N123 (Table 26). These analogs have 6-chloro- and 6-methyl-substituents in place of the 6-dimethylamino-substituent of pyrvinium iodide (see Figure 9).

Ethidium bromide is mutagenic in *Salmonella typhimurium* only after metabolic activation (McCann *et al.*, 1975; Fukunaga and Yielding, 1983), however, ethidium bromide produces petites in stationary- and growing-phase cells of yeast without exogenous metabolic activation. Ethidium

bromide binds reversibly to the mitochondrial DNA of stationary-phase cells by intercalation, and irreversibly in growing cells by covalent binding (Bastos and Mahler, 1974; Hixon *et al.*, 1975).

It has been suggested (Filipski, 1983; Marshall *et al.*, 1983) that ethidium bromide and an acridine derivative (4'-[9-acridinylamino] methanesulfon-*m*-anisidide) bind to a DNA nicking-closing enzyme, such as the topoisomerases I and II, by occupying the site of the enzyme that normally interacts with the DNA, and thereby preventing the nick from being sealed. Pyrvinium pamoate is a dipyrvinium salt that has two DNA binding sites per pyrvinium moiety, therefore this hypothesis may explain why pyrvinium pamoate is more efficient than pyrvinium iodide at inducing petites.

It has been reported that the mechanism of anthelmintic action of pyrvinium pamoate is the inhibition of transport and utilization of exogenous glucose in the pinworm, *Enterobius (Oxyuris) vermicularis* (Rollo, 1980). The petites induced by pyrvinium pamoate in strain D5 of yeast remained white throughout the tetrazolium overlay only when a high concentration of glucose (10%), rather than the concentration that is usually used (2%), was provided in the medium. Thus, pyrvinium pamoate may exert a biochemical effect, as well as a genetic effect, on the yeast cells. The reason for this change in characteristic, which occurs when the concentration of the carbon source is altered, has not yet been elucidated.

It is possible that the site of action of pyrvinium pamoate is the mitochondria of the parasite, and in fact, the mechanism of action of this drug may be the destruction of the mitochondrial DNA. Ethidium bromide was once used as an anthelmintic drug, and it is eminently

possible that the primary action of pyrvinium pamoate and ethidium bromide on helminths is the destruction of the mitochondrial DNA.

TABLE 24

Induction of aberrant colonies and respiratory-deficient, cytoplasmic variants (petites) in growing cells of strain D5 of *Saccharomyces cerevisiae* by ethidium bromide and pyrvinium pamoate

Treatment and Concentration (moles/l)	Exposure Time (hours)	Survival (%)	Total Colonies[a]	Colony Phenotype[b]						Growth on YEPG Medium[c]	Classification
				RP	RPW	R	RW	P	PW		
water (2.5%)	0	100	406	0	0	0	0	0	0	–	–
	0.5	100	376	0	0	2	1	0	1	(+)	grande
	1	100	222	0	0	0	0	0	0	–	–
	2	100	255	0	0	0	0	0	0	–	–
	3	100	649	0	0	2	0	1	0	(+)	grande
DMSO (2.5%)	0	101	410	0	1	0	0	0	0	(+)	grande
	0.5	141	530	0	0	0	1	2	0	(+)	grande
	1	315	646	0	1	0	0	1	1	(+)	grande
	2	147	375	0	0	0	0	0	0	–	–
	3	76	491	0	0	0	1	0	1	(+)	grande
ethidium bromide 2.5×10^{-5}	0	35	213	0	0	0	0	0	0	–	–
	0.5	55	311	0	0	1	0	0	0	(-)	petite
	1	153	471	1	0	1	0	0	0	(-)	petite
	2	36	93	0	0	0	0	0	0	–	–
	3	75	456	0	0	0	2	0	0	(-)	petite
pyrvinium pamoate 4×10^{-9}	0	52	314	0	0	0	1	0	1	(+)	grande
	0.5	26	144	0	0	0	0	0	1	(+)	grande
	1	153	471	0	0	3	0	0	0	(+)	grande
	2	46	138	0	0	0	0	0	0	–	–
	3	75	568	0	0	1	1	4	1	(+)	grande

TABLE 24 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours	Survival (%)	Total Colonies[a]	Colony Phenotype[b]						Growth on YEPG Medium[c]	Classification
				RP	RPW	R	RW	P	PW		
$4x10^{-7}$	0	79	480	0	1	2	0	0	1	(+)	grande
	0.5	24	89	0	0	1	0	0	0	(+)	grande
	1	134	412	0	0	0	1	0	0	(+)	grande
	2	32	82	0	0	1	0	0	0	(+)	grande
	3	72	543	0	1	0	0	1	0	(+)	grande
$4x10^{-5}$	0	47	288	0	1	1	1	1	0	(+)	grande
	0.5	25	93	0	0	0	0	0	0	(+)	grande
	1	86	264	0	0	0	1	1	0	(+)	grande
	2	36	106	0	0	1	0	0	0	(+)	grande
	3	56	423	0	0	0	0	0	0	–	–
$4x10^{-3}$	0	59	238	0	0	0	1	0	0	(+)	petite
	0.5	55	519	0	0	1	0	0	0	(+)	petite
	1	68	209	0	0	0	1	0	0	(+)	grande
	2	110	420	0	0	0	0	0	1	(-)	petite
	3	67	651	0	0	0	0	0	0	–	–

[a]The total number of surviving colonies on 4 plates of regular YEPD-agar (contains 2% dextrose) and 2 plates of fortified YEPD-agar (contains 10% dextrose).

[b]The abbreviations RP, RPW, R, RW, P, and PW refer to red-pink, red-pink-white, red, red-white, pink, and pink-white colonies, respectively.

[c]The colonies were classified as grande or petite by their ability or inability to grow on YEPG-agar (contains 2% glycerol as the carbon source).

TABLE 25

Induction of respiratory-deficient, cytoplasmic variants (petites) in growing cells of strain D5 of *Saccharomyces cerevisiae* by ethidium bromide and pyrvinium pamoate

Treatment and Concentration (moles/l)	Exposure Time (hours)	Dextrose Content of YEPD	Colony Color after Tetrazolium Overlay[a]			Petites[b] (%)	Survival (%)	Total Petites[c] (%)	Total Survival[d] (%)
			white	red	total				
water (2.5%)	0	2%	0	252	252	<0.4	100	<0.2 (0)	100 (406)
		10%	0	154	154	<0.6	100		
	0.5	2%	0	256	256	<0.4	100	<0.3 (0)	100 (376)
		10%	0	120	120	<0.8	100		
	1	2%	0	154	54	<0.6	100	<0.4 (0)	100 (222)
		10%	0	68	68	<1	100		
	2	2%	0	170	170	<0.6	100	0.4 (1)	100 (255)
		10%	1	84	85	1	100		
	3	2%	0	443	443	<0.2	100	0.2 (1)	100 (649)
		10%	1	205	206	0.5	100		
DMSO (2.5%)	0	2%	0	294	294	<0.3	117	0.2 (1)	101 (410)
		10%	1	115	116	0.9	75		
	0.5	2%	0	347	347	<0.3	136	<0.2 (0)	141 (530)
		10%	0	183	183	<0.6	152		
	1	2%	0	448	448	<0.2	291	<0.2 (0)	315 (646)
		10%	0	198	198	<0.5	291		
	2	2%	0	234	234	<0.4	138	<0.3 (0)	147 (375)
		10%	0	141	141	<0.7	166		
	3	2%	0	330	330	<0.3	74	0.2 (1)	76 (491)
		10%	1	160	161	0.6	78		

TABLE 25 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Dextrose Content of YEPD	Colony Color after Tetrazolium Overlay[a]			Petites[b] (%)	Survival (%)	Total Petites[c] (%)	Total Survival[d] (%)
			white	red	total				
ethidium bromide 2.5×10^{-5}	0	2%	0	171	171	<0.6	39	<0.5 (0)	35 (213)
		10%	0	42	42	<2	27		
	0.5	2%	239	0	239	100	53	100 (311)	55 (311)
		10%	72	0	72	100	60		
	1	2%	360	0	360	100	134	100 (471)	153 (471)
		10%	111	0	111	100	163		
	2	2%	61	0	61	100	36	100 (93)	36 (93)
		10%	32	0	32	100	38		
	3	2%	415	0	415	100	75	100 (567)	75 (567)
		10%	152	0	152	100	74		
pyrvinium pamoate 4×10^{-9}	0	2%	0	248	248	<0.4	56	<0.3 (0)	52 (314)
		10%	0	66	66	<2	43		
	0.5	2%	0	105	105	<1	23	0.7 (1)	26 (144)
		10%	1	38	39	3	32		
	1	2%	0	351	351	<0.3	130	<0.2 (0)	153 (471)
		10%	0	120	120	<0.8	176		
	2	2%	0	101	101	<1	48	<0.7 (0)	46 (138)
		10%	0	37	37	<3	44		
	3	2%	0	400	400	<0.2	72	0.2 (1)	75 (568)
		10%	1	167	168	0.6	82		

TABLE 25 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Dextrose Content of YEPD	Colony Color after Tetrazolium Overlay[a]			Petites[b] (%)	Survival (%)	Petites[c] (%)	Survival[d] (%)
			white	red	total				
$4x10^{-7}$	0	2%	0	351	351	<0.3	80	0.4 (2)	79 (480)
		10%	2	127	129	2	84		
	0.5	2%	0	61	61	<2	24	<1 (0)	24 (89)
		10%	0	28	28	<4	23		
	1	2%	0	334	334	<0.3	124	<0.2 (0)	134 (412)
		10%	0	78	78	<1	115		
	2	2%	0	54	54	<2	32	<1 (0)	32 (82)
		10%	0	28	28	<4	33		
	3	2%	0	414	414	<0.2	75	<0.2 (0)	72 (543)
		10%	0	129	129	<0.8	63		
$4x10^{-5}$	0	2%	0	217	217	<0.5	49	<0.4 (0)	47 (288)
		10%	0	71	71	<1	46		
	0.5	2%	0	64	64	<2	25	<1 (0)	25 (93)
		10%	0	29	29	<3	24		
	1	2%	0	198	198	<0.5	74	<0.4 (0)	86 (264)
		10%	0	66	66	<2	97		
	2	2%	0	78	78	<1	37	2 (2)	36 (106)
		10%	2	26	28	7	33		
	3	2%	69	238	307	22	55	34.5(146)	56 (423)
		10%	77	39	116	66	56		

TABLE 25 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Dextrose Content of YEPD	Colony Color after Tetrazolium Overlay[a]			Petites[b] (%)	Survival (%)	Petites[c] (%)	Survival[d] (%)
			white	red	total				
$4x10^{-3}$	0	2%	0	148	148	<0.7	59	0.8 (2)	59 (238)
		10%	2	88	90	2	58		
	0.5	2%	0	339	339	<0.3	53	0.2 (1)	55 (519)
		10%	1	179	180	0.6	60		
	1	2%	0	106	106	<0.9	69	0.5 (1)	68 (209)
		10%	1	102	103	1	61		
	2	2%	0	280	280	<0.4	110	6.9 (29)	110 (420)
		10%	29	111	140	21	110		
	3	2%	59	397	456	13	69	31.6 (206)	67 (651)
		10%	147	48	195	75.4	63		

[a] Colony color was scored 24 hours after the tetrazolium-overlay; white colonies were classified as petite and red colonies as grande. Colonies with red-pink, red-pink-white, red, red-white, pink, or pink-white phenotypes were sampled prior to the tetrazolium-overlay procedure and were classified as grande or petite by their ability or inability to grow on YEPG-agar (contains 2% glycerol as the carbon source), as described in Table 24.

[b] Wherever a petite count of zero appears, the percentage of petites is reported as less than the value corresponding to one petite colony.

[c] The figures in parentheses constitute the total number of petite colonies on 6 plates of YEPD-agar (the sum of the petite colonies on 4 plates of regular YEPD-agar [contains 2% dextrose] and 2 plates of fortified YEPD-agar [contains 10% dextrose]). Wherever a petite count of zero appears, the percentage of total petites is reported as less than the value corresponding to one petite colony.

[d] The figures in parentheses constitute the total number of surviving colonies on 6 plates of YEPD-agar (the sum of the surviving colonies on 4 plates of regular YEPD-agar and 2 plates of fortified YEPD-agar).

TABLE 26

Induction of respiratory-deficient, cytoplasmic variants (petites) in growing cells of strain N123 of *Saccharomyces cerevisiae* by ethidium bromide, pyrvinium pamoate, pyrvinium iodide, and the chloro- and methyl-analogs of pyrvinium iodide

Treatment and Concentration (moles/l)	Exposure Time (hours)	Dextrose Content of YEPD	Colony Color after Tetrazolium Overlay[a]			Petites[b] (%)	Survival (%)	Petites[c] (%)	Survival[d] (%)
			white	red	total				
water (2.5%)	1	2%	4	572	576	0.7	100	0.7 (8)	100 (1112)
		10%	4	532	536	0.8	100		
	2	2%	1	337	338	0.3	100	0.9 (6)	100 (653)
		10%	5	310	315	2	100		
	4	2%	1	236	237	0.4	100	0.7 (3)	100 (449)
		10%	2	210	212	0.9	100		
	8	2%	3	519	522	0.6	100	0.6 (7)	100 (1079)
		10%	4	545	549	0.7	100		
DMSO (2.5%)	1	2%	3	500	503	0.6	87	0.7 (7)	88 (974)
		10%	4	467	471	0.8	88		
	2	2%	5	329	334	2	99	1.5 (10)	100 (655)
		10%	5	316	321	2	102		
	4	2%	2	239	241	0.8	102	0.4 (2)	105 (470)
		10%	0	229	229	<0.4	108		
	8	2%	3	610	613	0.5	117	0.7 (8)	108 (1165)
		10%	5	547	552	0.9	101		

TABLE 26. (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Dextrose Content of YEPD	Colony Color after Tetrazolium Overlay[a]			Petites[b] (%)	Survival (%)	Petites[c] (%)	Survival[d] (%)
			white	red	total				
ethidium bromide $2.5x10^{-5}$	1	2%	376	88	464	81.0	81	79.2 (781)	89 (986)
		10%	405	117	522	77.6	97		
	2	2%	353	49	402	87.8	119	82.4 (626)	116 (760)
		10%	273	85	358	76.3	114		
	4	2%	524	62	586	89.4	112	91.0(1055)	117 (1160)
		10%	531	43	574	92.5	123		
	8	2%	365	2	367	99.5	32	99.7 (701)	30 (703)
		10%	336	0	336	100	28		
pyrvinium pamoate $4x10^{-4}$	1	2%	6	428	434	1	75	0.8 (7)	78 (864)
		10%	1	429	430	0.2	80		
	2	2%	4	274	278	1	82	1 (6)	89 (579)
		10%	2	299	301	0.7	96		
	4	2%	14	481	495	2.8	95	3.7 (37)	101 (994)
		10%	23	476	499	4.6	107		
	8	2%	398	287	685	58.1	60	60.2 (797)	56 (1325)
		10%	399	241	640	62.3	53		

TABLE 26 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Dextrose Content of YEPD	Colony Color after Tetrazolium Overlay[a]			Petites[b] (%)	Survival (%)	Petites[c] (%)	Survival[d] (%)
			white	red	total				
pyrvinium iodide $1x10^{-4}$	1	2%	5	547	552	0.9	96	0.7 (8)	97 (1079)
		10%	3	524	527	0.6	98		
	2	2%	4	352	356	1	105	0.6 (4)	108 (705)
		10%	0	349	349	<0.3	111		
	4	2%	24	1245	1269	1.9	107	1.7 (42)	112 (2510)
		10%	18	1223	1241	1.4	117		
	8	2%	34	320	354	9.6	24	13 (90)	23 (688)
		10%	56	278	334	17	22		
$2x10^{-4}$	1	2%	6	500	506	1	88	1.0 (10)	88 (977)
		10%	4	467	471	0.8	88		
	2	2%	1	324	325	0.3	96	0.3 (2)	98 (637)
		10%	1	311	312	0.3	99		
	4	2%	20	1235	1255	1.6	106	1.5 (36)	107 (2412)
		10%	16	1141	1157	1.4	109		
	8	2%	60	416	476	13	23	14.3 (138)	22 (966)
		10%	78	412	490	16	22		

TABLE 26 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Dextrose Content of YEPD	Colony Color after Tetrazolium Overlay[a]			Petites[b] (%)	Survival (%)	Petites[c] (%)	Survival[d] (%)
			white	red	total				
$4x10^{-4}$	1	2%	1	495	496	0.2	86	0.2 (2)	87 (969)
		10%	1	472	473	0.2	88		
	2	2%	3	527	530	0.6	78	0.5 (5)	84 (1093)
		10%	2	561	563	0.4	89		
	4	2%	21	1618	1639	1.3	105	1.5 (47)	108 (3199)
		10%	26	1534	1560	1.7	112		
	8	2%	88	354	442	20	21	18.8 (163)	20 (868)
		10%	75	351	426	18	19		
$8x10^{-4}$	1	2%	7	398	405	2	70	1.2 (10)	73 (808)
		10%	3	400	403	0.7	75		
	2	2%	9	599	608	2	90	1.3 (16)	93 (1218)
		10%	7	603	610	1	97		
	4	2%	37	1570	1607	2.3	103	2.0 (61)	105 (3123)
		10%	24	1492	1516	1.6	108		
	8	2%	42	334	376	11	18	10 (72)	16 (693)
		10%	30	287	317	9.5	14		

TABLE 26 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Dextrose Content of YEPD	Colony Color after Tetrazolium Overlay[a]			Petites[b] (%)	Survival (%)	Petites[c] (%)	Survival[d] (%)
			white	red	total				
6-chloro-analog of pyrvinium iodide $2x10^{-4}$	1	2%	2	553	555	0.4	96	0.7 (8)	103 (1143)
		10%	6	582	588	1	110		
	2	2%	5	408	413	1	122	0.9 (7)	119 (777)
		10%	2	362	364	0.6	116		
	4	2%	5	552	557	0.9	107	1.2 (12)	102 (1005)
		10%	7	441	448	2	96		
	8	2%	42	278	320	13	22	12 (72)	20 (598)
		10%	30	248	278	11	18		
6-methyl-analog of pyrvinium iodide $2x10^{-4}$	1	2%	4	444	448	0.9	78	1 (9)	81 (900)
		10%	5	447	452	1	84		
	2	2%	0	193	193	<0.5	57	1 (4)	61 (396)
		10%	4	199	203	2	64		
	4	2%	6	326	332	2	64	1.9 (13)	69 (682)
		10%	7	343	350	2	75		
	8	2%	68	856	924	7.4	10	6.8(124)	9 (1826)
		10%	56	846	902	6.2	9		

TABLE 26 (continued)

Treatment and Concentration (moles/l)	Exposure Time (hours)	Dextrose Content or YEPD	Colony Color after Tetrazolium Overlay[a]			Petites[b] (%)	Survival (%)	Petites[c] (%)	Survival[d] (%)
			white	red	total				

[a]Colony color was scored 24 hours after the tetrazolium-overlay procedure; white colonies were classified as petite and red colonies as grande.

[b]Wherever a petite count of zero appears, the percentage of petites is reported as less than the value corresponding to one petite colony.

[c]The figures in parentheses constitute the total number of petite colonies on 10 plates of YEPD-agar (the sum of the petite colonies on 5 plates of regular YEPD-agar [contains 2% dextrose] and 5 plates of fortified YEPD-agar [contains 10% dextrose]). Wherever a petite count of zero appears, the percentage of total petites is reported as less than the value corresponding to one petite colony.

[d]The figures in parentheses constitute the total number of surviving colonies on 10 plates of YEPD-agar (the sum of the surviving colonies on 5 plates of regular YEPD-agar and 5 plates of fortified YEPD-agar).

ARTICLE VI

The Genetic Activity of Pyrvinium Pamoate in Mammalian Cells *In Vivo:* Induction of Micronuclei in Mouse Bone Marrow and Nuclear Aberrations in Mouse Colon

ABSTRACT

Pyrvinium pamoate, an anthelmintic drug that is a mutagen in bacteria and yeast, is active genetically in mammalian cells *in vivo*. Preliminary results indicate that elevated levels of micronuclei were induced in the bone marrow cells of ♀B6C3F1 mice after an intraperitoneal injection of pyrvinium pamoate, with dimethylsulfoxide (DMSO) as the vehicle. Peak levels of micronuclei were attained at 48 hours after the treatment, and control levels were reached by 72 hours. Low levels (only about 2-fold greater than the values for the DMSO-treated and untreated controls) of nuclear aberrations were observed in the colon cells of ♂C57BL mice after the oral administration of pyrvinium pamoate (1.2 mg/kg) that was dissolved in DMSO and oral suspensions of pyrvinium pamoate (Vanquin® and Pyr-Pam®) that are available commercially.

INTRODUCTION

Pyrvinium pamoate has been demonstrated to be a mutagen in bacteria under various conditions. Some medical grades of the drug require metabolic activation to cause base-substitution and frameshift mutations in *Salmonella typhimurium* (MacPhee and Podger, 1977; Lake and de la Iglesia, 1981), whereas other batches of the drug do not require such activation to be mutagenic (Cortinas de Nava *et al.*, 1983; Hennig *et al.*, 1984). The dipyrvinium salt, pyrvinium pamoate, and the monopyrvinium salts, pyrvinium chloride and pyrvinium iodide, are also mutagenic and recombinogenic in haploid (XV185-14C) and diploid (D5) strains, respectively, of the yeast *Saccharomyces cerevisiae* (Galindo *et al.*, 1979; Hennig *et al.*, 1981; Mehta *et al.*, 1982).

The mutagenicity of pyrvinium pamoate in microbial systems can be considered to be an indication that the drug may also damage the genetic material of mammalian cells. To assess the effects of pyrvinium pamoate in mammalian cells, *in vitro* assays were carried out by Lake and de la Iglesia (1981) in C3H10T½ mouse cells for cell transformation, in Chinese hamster ovary (CHO) cells for chromosomal aberrations, and in CHO cells for sister-chromatid exchange, with or without the addition of the S9 fraction from mammalian liver. Over the cytotoxicity-limited dose range of pyrvinium pamoate that was used (0.01-0.78 µg/ml in the culture medium), significant increases ($p>0.1$) in cell transformation, chromosome aberrations, or sister-chromatid exchanges were not observed, whereas the positive controls (7,12-dimethylbenz[*a*]anthracene, dimethylnitrosamine, and cyclophosphamide) caused significant increases ($p<0.001$) in each of these genetic endpoints.

Pyrvinium pamoate has also been studied *in vivo* by the analyses of urine samples from humans and mice that were treated with the drug. The 24-hour urine samples from the mice that had been treated with a single, oral dose of pyrvinium pamoate (100 mg/kg) were not mutagenic in strains TA98 and TA100 of *Salmonella typhimurium* (Lake and de la Iglesia, 1981). Similarly, the 24-hour urine specimens from the mice that had been treated with lower oral doses (0.75 mg/kg, 7.5 mg/kg, and 75 mg/kg) of pyrvinium pamoate were not mutagenic in strain TA100 (Cortinas de Nava *et al.*, 1983). The 24-hour specimens of human urine, collected after a single, oral dose (10 mg/kg) of pyrvinium pamoate, were not mutagenic in strains TA98 and TA100 of *Salmonella typhimurium* (Lake and de la Iglesia, 1981).

Pyrvinium pamoate is a mutagen in bacteria and yeast. In Canada, pyrvinium pamoate is an over-the-counter anthelmintic drug, therefore many children are exposed to this drug on a daily basis. Although the results of the *in vitro* mammalian assays and the mammalian urine analyses (Lake and de la Iglesia, 1981; Cortinas de Nava *et al.*, 1983) suggest that the drug is not hazardous, it is important to evaluate pyrvinium pamoate *in vivo* in mammalian assays that reflect both the metabolism and the tissue specificity that this anthelmintic drug would undergo in humans, at doses in the therapeutic range. This type of evaluation was carried out in mice, *in vivo*, and involved the analyses of the erythropoietic tissue and the cells at the bottom of the crypts of the colon to determine whether or not these replicating cells are damaged by pyrvinium pamoate. The relevance of the colon assay was increased by the use of dosage forms (oral suspensions) of pyrvinium pamoate that are available commercially in Canadian pharmacies.

MATERIALS AND METHODS

Animals

For the bone marrow assay, 6-week-old ♀B6C3F1 mice were used, and 9-week-old ♂C57BL mice were used for the colon studies. The mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, U.S.A.). The animals that were used for the colon studies were denied access to food and water for 4 hours prior to the gavage procedure.

Drugs and Solutions

Pyrvinium pamoate was obtained from Parke-Davis Canada Incorporated, and 1,2-dimethylhydrazine·2HCl and 7,12-dimethylbenz[α]anthracene were purchased from the Sigma Chemical Company (Saint Louis, Missouri, U.S.A.).

Stock solutions of pyrvinium pamoate (0.16 mg/ml and 0.15 mg/ml), 1,2-dimethylhydrazine·2HCl (2 mg/ml and 0.6 mg/ml), and 7,12-dimethylbenz[α]anthracene (5 mg/ml) were prepared in dimethylsulfoxide (DMSO). The solutions were diluted with DMSO when required. The oral suspensions of pyrvinium pamoate (Vanquin® from Parke-Davis Canada Incorporated and Pyr-Pam® from ICN Canada Limited) were used as supplied by the manufacturers.

Induction of Micronuclei in Bone Marrow

The doses of pyrvinium pamoate (0.2 mg/kg, 0.4 mg/kg, and 0.8 mg/kg), 7,12-dimethylbenz[α]anthracene (25 mg/kg), and 1,2-dimethylhydrazine·2HCl (10 mg/kg) were administered to ♀B6C3F1 mice by an intraperitoneal injection (0.1 ml/mouse of the appropriate stock solution, or dilution, that had been prepared in DMSO). The controls were

DMSO-treated (0.1 ml/mouse) and untreated mice.

The animals were sacrificed by cervical dislocation, carbon dioxide (CO_2) gas, or exposure to CO_2 vapors from dry ice. Bone marrow samples from a femur were taken at 24, 48, and 72 hours after the intraperitoneal injection. Each bone marrow sample was placed onto a microscope slide, mixed with a drop of fetal calf serum, fixed in methanol, and stained with 5% Giemsa, as described by Heddle *et al.* (1984). As a result of the Giemsa stain, white blood cells were deep blue, red blood cells were pink, and polychromatic erythrocytes were light blue and tinged with purple.

Induction of Nuclear Aberrations in Mouse Colon

The ♂C57BL mice received the suspensions of pyrvinium pamoate (Vanquin® and Pyr-Pam®) by gavage (0.2 ml/mouse). The doses of pyrvinium pamoate (0.6 mg/kg and 1.2 mg/kg) and 1,2-dimethylhydrazine·2HCl (2.4 mg/kg and 4.8 mg/kg) were administered by oral intubation (0.2 ml/mouse of the appropriate stock solution, or dilution, that had been prepared in DMSO). The controls were DMSO-treated (0.2 ml/mouse) and untreated mice.

The animals were sacrificed by cervical dislocation 24 hours after the treatment, and their colons were excised and flushed with phosphate-buffered saline (pH 7.2). Each colon was cut open from the cecum to the anus and rolled from the proximal to the distal end (Wargovich *et al.*, 1983a). The tissue samples were fixed in 10% buffered formalin phosphate, processed, and histological sections (6 μm thick) were cut from the paraffin-embedded tissue. The tissue preparations were stained by the Feulgen method (Kiernan, 1981) and a fast green counterstain. In

the Feulgen reaction, the modified aldehydes in the Schiff reagent react with the primary amino groups, which have been liberated from the DNA and chromatin by the hydrolyzing action of concentrated hydrochloric acid, to form Schiff bases. The longitudinal columns of the crypts were scanned for nuclear aberrations and all forms of nuclear damage were scored.

RESULTS AND DISCUSSION

Micronuclei arise from chromosomal fragments that are not incorporated into the daughter nuclei at the time of cellular division. Chromosome breakage is a lethal event since chromosomal fragments usually lack centromeres and remain in the cytoplasm upon cellular division. These acentric fragments are incorporated as micronuclei that can be detected at interphase and are lost from the daughter nuclei upon mitosis. The micronucleus assay detects chromosome aberrations and chromosomal breakage, *in vivo*, in somatic cells that are active mitotically. The test can be used to detect chromosome-breakage events that are induced by chemicals, either *in vitro* (in cells in culture) or *in vivo* (in animal bone marrow) (Heddle, 1973; Schmid, 1975).

The micronucleus assay involves the analysis of polychromatic erythrocytes because they are the most convenient population of cells in the bone marrow in which to study micronuclei. Polychromatic erythrocytes are the immediate end-product of a series of cellular divisions; they do not divide further and gradually mature into

normochromatic erythrocytes. The nucleus is expelled about 8-12 hours after the last mitotic division that precedes the formation of an erythrocyte, therefore polychromatic erythrocytes do not contain DNA. The staining properties of polychromatic erythrocytes are due to the presence of ribosomal RNA, which is present for approximately 24 hours after the cell is formed. The cells lose this RNA as they mature into normochromatic erythrocytes. Giemsa's stain, which utilizes nucleophilic dyes that are taken up readily by micronuclei, is preferred for distinguishing between white blood cells, red blood cells, and polychromatic erythrocytes. A good resolution of the staining characteristics is essential because the maturation of a polychromatic erythrocyte into a normochromatic erythrocyte involves a continual gradation in staining properties. Micronuclei are easy to identify because they stain intensely.

Polychromatic erythrocytes and mature erythrocytes do not contain nuclei because the nuclei are expelled approximately 8-12 hours after the formation of the erythrocytes, however, micronuclei remain for about 24 hours. This time sequence for the expulsion of nuclei and the retention of micronuclei must be taken into account in the bone marrow assay so that the treatment and sampling times can be scheduled accordingly. The reasons for the retention of micronuclei are not known. Damaged erythrocytes, such as micronucleated red blood cells, normally are not found in the circulation because they are filtered out by the spleen.

The strains of mice that are used in the bone marrow assay must be responsive to the induction of metabolizing enzymes by the administration of aromatic hydrocarbons, and the aryl hydrocarbon hydroxylases of

the hepatic cytochrome P-450 system can be induced readily in certain strains of mice (Kouri and Nebert, 1977). B6C3F1 mice, the F_1 generation of a cross between inducible parents (♀C57BL/6 x ♂C3H), are a genetically uniform but outcrossed stock (Heddle and Salamone, 1981).

Drugs can be dissolved in saline, corn oil, ethanol, or DMSO and should be administered by the most appropriate route (oral, intraperitoneal, etc.), if possible. Each drug should be tested at the highest dose possible to avoid false negatives. The highest dose of pyrvinium pamoate that would permit 24 hours of survival after an intraperitoneal injection was 0.8 mg/kg. DMSO was used as the solvent because pyrvinium pamoate is insoluble in saline and corn oil, and only sparingly soluble in ethanol. To avoid the toxic and mutagenic effects of DMSO, not more than 0.15 ml/20-g mouse should be injected intraperitoneally; this dose does not result in increases in the control levels of micronuclei (Heddle and Salamone, 1981). The spontaneous levels of micronuclei (Table 27) in the untreated mice, the DMSO-treated mice, and the untreated and DMSO-treated animals combined (1.6 micronuclei/1000 polychromatic erythrocytes) are within the range that was expected for the negative controls (Heddle and Salamone, 1981). Based on these control values, pyrvinium pamoate can be considered to be clastogenic in the bone marrow. All three doses of the drug (0.2 mg/kg, 0.4 mg/kg, and 0.8 mg/kg) induced levels of micronuclei that exceeded the control values by at least 2-fold. Peak levels of induction appear to be attained at approximately 48 hours after the intraperitoneal injection and control levels were reached at 72 hours (Table 27).

The statistical evaluation of a response as negative or positive in the bone marrow assay depends on the experimental design, which

includes factors such as the number of mice used, the number and range of sampling times, the number of polychromatic erythrocytes scored per mouse, the number and range of doses used, and the route of administration. In a comparison with the positive controls, 7,12-dimethylbenz-[*a*]anthracene (Heddle and Salamone, 1981) and 1,2-dimethylhydrazine-·2HCl, which induced approximately 8-fold increases in the level of micronuclei (Table 27), pyrvinium pamoate demonstrated genetic activity *in vivo*. The methods of euthanasia (cervical dislocation, CO_2 gas, or CO_2 vapors from dry ice) did not produce visible artefacts or adverse effects in the bone marrow assay (Table 27).

The positive results that were obtained in the bone marrow assay suggest that pyrvinium cations that cross into the blood stream from the gastrointestinal tract, after an oral administration of pyrvinium pamoate, also could induce chromosomal damage. It is believed that pyrvinium pamoate does not cross the barrier between the gastrointestinal tract and the blood stream (Rollo, 1980), however, it is known that monopyrvinium salts can cross this barrier (Hales and Welch, 1953). The colons of Sprague-Dawley rats were exposed, *in vitro*, to pyrvinium pamoate for 15 minutes and the epithelial cells were removed with EDTA; fluorescence microscopy showed that these cells had taken up the drug (P. Dickie, unpublished data).

Whether or not pyrvinium pamoate can concentrate in the epithelial cells of the gastrointestinal tract, particularly in the crypts of the colon where cell division takes place at a high rate, after exposure to the drug, *in vivo*, was not known. The fluorescent properties of pyrvinium pamoate facilitated the tracing of the tissue distribution of the drug in the gastrointestinal tract. Pyrvinium pamoate (dissolved

in DMSO) was administered to Sprague-Dawley rats by oral intubation, the rats were killed with CO_2 gas 24 hours after the treatment, and several gastrointestinal organs (stomach, small intestine, cecum, colon, and rectum) were prepared for freeze-dry sectioning. The freeze-dried sections were scanned under a fluorescence microscope and it was evident that pyrvinium pamoate penetrates into the epithelial lining of the stomach and the bottom of the crypts in colon cells (Figure 11), but it does not appear to enter the epithelial lining of the small intestine (P. Dickie and U.G.G. Hennig, unpublished data).

The bone marrow micronucleus test detects, rapidly and reproducibly, the genetic activity of drugs in erythropoietic tissue, with chromosome breakage as the genetic endpoint. It is important to detect similar responses in other tissues and organs since certain organs and cells may be in constant and specific contact with a particular drug. For example, pyrvinium pamoate appears to be in constant contact with the highly proliferative, epithelial cell population in the lining of the colon.

The colon carcinogen 1,2-dimethylhydrazine·2HCl induces nuclear aberrations in the proliferating compartments of the crypts in the colonic epithelium, and this damage may be analogous to the induction of micronuclei in erythropoietic cells. The nuclear abnormalities (expressed as total aberrations/crypt) caused by 1,2-dimethylhydrazine·2HCl reach a maximum value 24 hours after the intraperitoneal injection, and then the frequency of aberrations/crypt declines and reaches control levels 4 days after the treatment (Wargovich *et al.*, 1983b).

Values for negative controls, such as DMSO, have been reported to be in the range of 0.04 - 0.2 nuclear aberrations/crypt, with an

average (mean and standard error) of 0.12 ± 0.06 (Wargovich *et al.*, 1983a). The oral suspensions of pyrvinium pamoate, Vanquin® and Pyr-Pam®, elicited an approximately 2-fold increase in the frequency of nuclear aberrations/crypt when compared to the DMSO-treated and untreated controls (Table 28). Pyrvinium pamoate (dissolved in DMSO) appears to elicit a distinctly positive effect only at the higher dose (1.2 mg/kg). This evaluation has been made with the criterion for an unequivocal, significant increase in colon damage being a 4-fold increase in nuclear aberrations/crypt over the control value (Wargovich *et al.*, 1983a). All morphological forms of nuclear damage (i.e., disintegrated nuclei, karyorrhectic nuclei, micronuclei, pyknotic nuclei, cytolysosomes, vacuolated bodies, and mitotic figures) were included in the assessment of nuclear aberrations. The differences between the crypts of the DMSO-treated and the 1,2-dimethylhydrazine·2HCl-treated mice are quite evident (Figure 12).

The colon samples were taken 24 hours after the treatment because the colonic nuclear events caused by the positive control, 1,2-dimethylhydrazine·2HCl, reach a peak at 24 hours (Wargovich *et al.*, 1983b). It is possible that the peak level of nuclear lesions that are caused by pyrvinium pamoate is not observed at 24 hours, and an indication of this is evident in the bone marrow assay, in which 48 hours was required to attain peak levels of micronuclei (Table 27). To be certain that pyrvinium pamoate causes nuclear aberrations in the colon cells, it would be necessary to increase the number and range of the doses of the drug, analyze more crypts/mouse, and use a greater number and range of sampling times.

The bone marrow micronucleus assay is a relatively simple and

rapid *in vivo* mammalian screening system that can be used in conjunction with bacterial and yeast assays to assess the probability of an effect of a drug in animals. The intestinal carcinogen 1,2-dimethylhydrazine·2HCl induces acute, nuclear defects in the cells of the proliferative compartment of the colonic crypts, and these nuclear aberrations may be due to chromosome aberrations analogous to those in the bone marrow studies. Pyrvinium pamoate, which is a mutagen in bacteria and yeast, also induces micronuclei in the bone marrow, and this indicates that the drug is active genetically in animals. The level of damage (nuclear aberrations/crypt) that was caused by pyrvinium pamoate in the colon cells is only about 2-fold greater than the control values, whereas the increases for 1,2-dimethylhydrazine·2HCl are approximately 75-fold. The results of these *in vivo* mammalian assays suggest that pyrvinium pamoate should be viewed as an anthelmintic drug with the potential for genetic activity in the human system.

TABLE 27

Induction of micronuclei in the bone marrow of ♀B6C3F1 mice by the intraperitoneal injection of pyrvinium pamoate

Drug[a]	Dose (mg/kg body weight)	Time (hours)	Method of Euthanasia	Micronuclei per 500 RBCs[b]	Micronuclei per 1000 PCEs[c]	Micronuclei per 1000 PCEs ($\bar{x}$ ± SE)[d]
untreated	--	48	cervical dislocation	0 3	1 1	1
dimethylsulfoxide	--	48	cervical dislocation	0 0 1	0 2 4	2.0 ± 1.2
pyrvinium pamoate	0.2	24	cervical dislocation	0 3 1 1	2 2 5 7	4.0 ± 1.2
		48	cervical dislocation	1 2 1 2	2 3 4 5	3.5 ± 0.6
			carbon dioxide (CO_2) gas	2 3 4 4	0 2 4 10	4.0 ± 2.2

TABLE 27 (continued)

Drug[a]	Dose (mg/kg body weight)	Time (hours)	Method of Euthanasia	Micronuclei per 500 RBCs[b]	Micronuclei per 1000 PCEs[c]	Micronuclei per 1000 PCEs ($\bar{x}$ ± SE)[d]
		72	cervical dislocation	0 2	1 1	1
	0.4	24	cervical dislocation	5 1 0 4	3 4 5 5	4.2 ± 0.5
		48	cervical dislocation	1 4 0 6	4 6 8 11	7.2 ± 1.5
			carbon dioxide (CO_2) gas	1 4 0 0	2 5 7 8	5.5 ± 1.3
		72	cervical dislocation	2 1 1 0	1 2 2 3	2.0 ± 0.4

TABLE 27 (continued)

Drug[a]	Dose (mg/kg body weight)	Time (hours)	Method of Euthanasia	Micronuclei per 500 RBCs[b]	Micronuclei per 1000 PCEs[c]	Micronuclei per 1000 PCEs ($\bar{x} \pm SE$)[d]
	0.8	24	cervical dislocation	3	3	
				4	5	
				4	5	
				3	6	
				0	7	6.6 ± 0.6
				3	7	
				6	7	
				4	8	
				5	8	
				5	10	
7,12-dimethyl-benz[a]anthracene	25	48	cervical dislocation	2	10	
				1	11	
				2	11	
				3	11	12.5 ± 0.9
				2	12	
				6	13	
				7	14	
				10	18	
		72	cervical dislocation	4	8	8.5 ± 0.5
				4	9	

TABLE 27 (continued)

Drug[a]	Dose (mg/kg body weight)	Time (hours)	Method of Euthanasia	Micronuclei Per 500 RBCs[b]	Micronuclei per 1000 PCEs[c]	Micronuclei per 1000 PCEs ($\bar{x}$ ± SE)[d]
1,2-dimethyl-hydrazine·2HCl	10	72	cervical dislocation	0	3	
				8	10	
				0	13	15.0 ± 4.7
				10	18	
				20	31	
			CO_2 vapors from dry ice	4	9	
				9	11	
				5	13	12.2 ± 1.5
				5	16	

[a] The appropriate dose of each drug was delivered in 0.1 ml of the vehicle, dimethylsulfoxide. The control animals treated with the vehicle received 0.1 ml of dimethylsulfoxide.

[b] The number of micronuclei/500 red blood cells observed for each individual mouse.

[c] The number of micronuclei/1000 polychromatic erythrocytes observed for each individual mouse.

[d] The calculated values (mean and standard error of the mean) for each treatment. One slide, which we believed to be mislabeled, was omitted from the calculations for the dimethylsulfoxide-treated mice.

TABLE 28

Oral administration of pyrvinium pamoate to ♂C57BL mice and the analysis of colon cells for induced nuclear aberrations 24 hours after the treatment

Drug	Dose[a] (mg/kg body weight)	Number of Nuclear Aberrations/Number of Crypts Analyzed[b]	Nuclear Aberrations per Crypt ($\bar{x} \pm SE$)[c]
untreated	--	0/40 (0) 0/40 (0) 1/31 (0.032) 1/21 (0.048) 2/40 (0.050)	0.026 ± 0.011
dimethylsulfoxide	--	0/40 (0) 0/40 (0) 1/40 (0.025) 1/40 (0.025) 1/40 (0.025)	0.015 ± 0.006
Vanquin® oral suspension (15 mg pyrvinium pamoate/ml)	equivalent to 12 mg pyrvinium pamoate	0/40 (0) 0/40 (0) 1/40 (0.025) 3/50 (0.060) 6/50 (0.120)	0.041 ± 0.023
Pyr-Pam® oral suspension (15 mg pyrvinium pamoate/ml)	equivalent to 12 mg pyrvinium pamoate	0/40 (0) 1/40 (0.025) 1/40 (0.025) 2/45 (0.044) 3/40 (0.075)	0.034 ± 0.012

TABLE 28 (continued)

Drug	Dose[a] (mg/kg body weight)	Number of Nuclear Aberrations/Number of Crypts Analyzed[b]	Nuclear Aberrations per Crypt ($\bar{x}$ ± SE)[c]
pyrvinium pamoate	0.6	0/40 (0) 0/40 (0) 1/60 (0.017) 1/39 (0.026)	0.011 ± 0.006
	1.2	1/60 (0.017) 1/40 (0.025) 3/40 (0.075) 38/100(0.430)	0.137 ± 0.099
1,2-dimethyl-hydrazine·2HCl	2.4	69/70 (0.986) 41/40 (1.025) 65/60 (1.083) 47/40 (1.175) 116/60 (1.933)	1.240 ± 0.176
	4.8	46/47 (0.979) 40/36 (1.111) 75/41 (1.829) 107/48 (2.229) 79/32 (2.469)	1.723 ± 0.296

TABLE 28 (continued)

Drug	Dose[a] (mg/kg body weight)	Number of Nuclear Aberrations/Number of Crypts Analyzed[b]	Nuclear Aberrations per Crypt ($\bar{x}$ ± SE)[c]

[a] The doses of the Vanquin® (Parke-Davis Canada Incorporated) and Pyr-Pam® (ICN Canada Limited) oral suspensions (0.2 ml/mouse), and the quantities of pyrvinium pamoate in each of these dosage forms were calculated from the labeled strengths on the packages.

[b] The figures in parentheses represent the number of nuclear aberrations/crypt calculated for each individual mouse.

[c] The calculated values (mean and standard error of the mean) for each treatment.

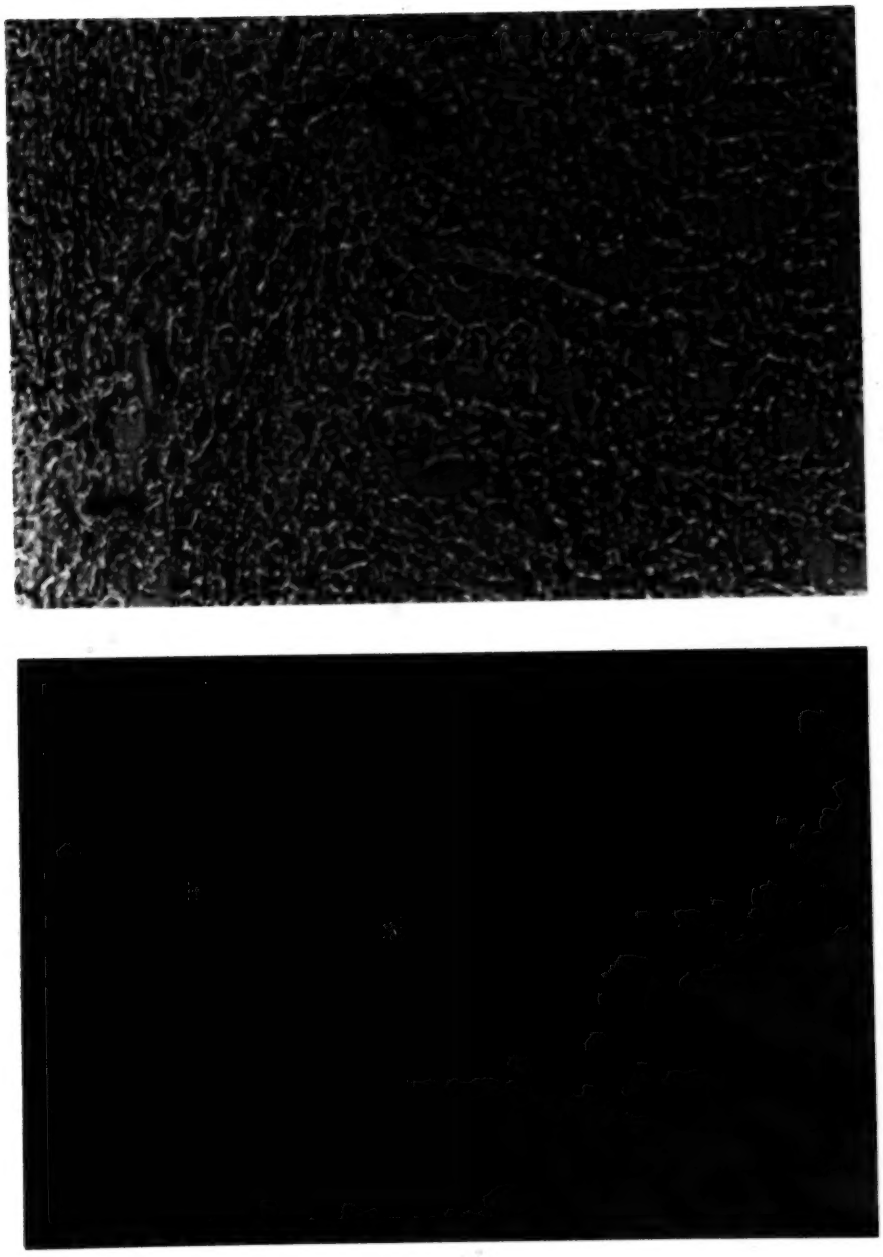


FIGURE 11. A longitudinal section of the colon that was excised from a Sprague-Dawley rat 24 hours after an oral intubation of pyrvinium pamoate (10 mg/0.5 ml DMSO). Top: Transmitted light; Bottom: Epifluorescence (N-2 rhodamine filter block, with an excitation range of 530-560 nm and a 580-nm emission filter). Magnification of 640X. (P. Dickie and U.G.G. Hennig, unpublished data).

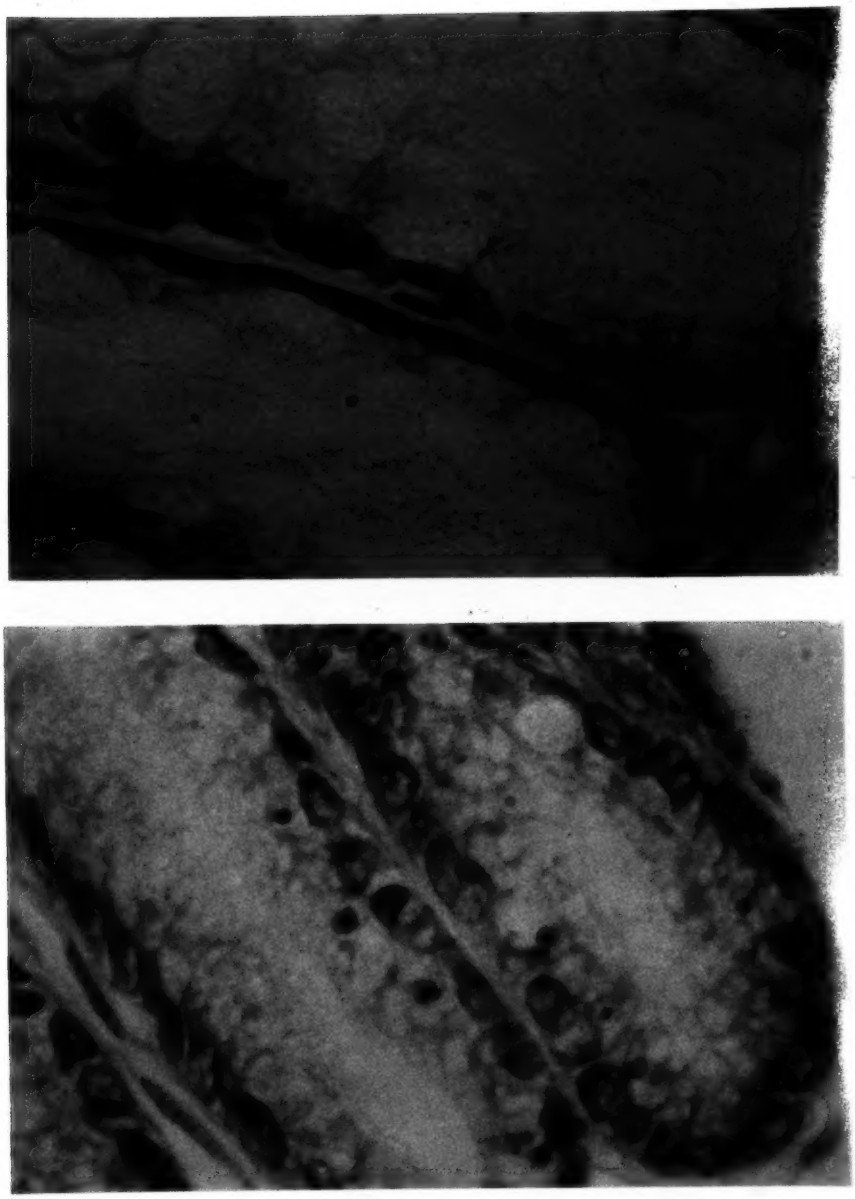


FIGURE 12. A comparison of histological sections that show the colonic crypts of vehicle-treated and carcinogen-treated ♂C57BL mice (Feulgen-fast green stain). Top: Section 24 hours after an oral intubation of 0.2 ml of DMSO; Bottom: Section 24 hours after an oral intubation of 1,2-dimethylhydrazine·2HCl (0.12 mg/0.2 ml DMSO, which was equivalent to 4.8 mg/kg body weight). Magnification of 640X.

SUMMARY AND CONCLUSIONS

The fact that pyrvinium pamoate is mutagenic in bacteria and yeast is a matter of concern since many children are exposed to this anthelmintic drug on a daily basis. An examination of the purity, the molecular action, and the tissue specificity of pyrvinium pamoate was of importance in the evaluation of the potentially harmful effects of the drug.

1. Light-catalyzed degradation, which can arise as a consequence of the improper storage of the drug, was not responsible for the genetic activity that was observed with the USP reference standard and the medical grades of pyrvinium pamoate that were tested. In fact, the genetic activity of the drug appeared to decrease gradually as a consequence of photodegradation.

2. The monopyrvinium salts, pyrvinium chloride and pyrvinium iodide, were more toxic and exhibited stronger genetic activity when they were compared to equimolar concentrations of pyrvinium pamoate. The differences in solubility, and thus the availability for cellular uptake, appears to be an important factor in the variation in the degree of genetic activity that was exhibited by the individual pyrvinium salts.

3. The enhanced genetic activity of certain medical grades of pyrvinium pamoate may be due to one or more of the numerous impurities that were revealed in the TLC analyses.

4. The highly mutagenic medical grades of pyrvinium pamoate were nearly as active genetically as pyrvinium chloride and pyrvinium iodide.

5. The absence of a mutagenic response to monopotassium pamoate indicates that the pyrvinium moiety itself is responsible for the genetic activity of pyrvinium pamoate.

6. Fluorescence high pressure liquid chromatography has revealed the existence of a discrepancy between the peak height of pyrvinium and the relative quantity of pyrvinium pamoate that had been injected. This discrepancy applies to the highly mutagenic medical grades of pyrvinium pamoate, and this has been postulated to be due to the presence of monopyrvinium salts.

7. An incomplete chemical reaction between a monopyrvinium salt and a pamoate salt during the synthesis of pyrvinium pamoate would account for the presence of monopyrvinium salts in certain medical grades of pyrvinium pamoate.

8. In the bacterium *Salmonella typhimurium*, highly purified pyrvinium pamoate is a promutagen that is converted into a mutagen by mammalian liver microsomes. The medical grades of pyrvinium pamoate that were highly mutagenic in yeast did not require metabolic activation to be mutagenic in bacteria.

9. Pyrvinium pamoate, pyrvinium iodide, and the 6-chloro-analog of pyrvinium iodide induced frameshift mutations and transitions in yeast, whereas the 6-methyl-analog of pyrvinium iodide induced frameshift mutations, transitions, and transversions. It is probable that the hydroxylation of the methyl-substituent of the 6-methyl-analog results in a mutagen that is as active as pyrvinium iodide itself.

10. The pyrvinium moiety may have an additional active site, which could be the cationic site at the methylated ring nitrogen. This theory is supported by the diminished, but not abolished, genetic activity of the 6-chloro-analog of pyrvinium iodide.

11. Cytoplasmic, respiratory-deficient variants were induced in *Saccharomyces cerevisiae* with the pyrvinium salts and the analogs. Pyrvinium pamoate was more efficient than pyrvinium iodide and its analogs at inducing petites.

12. Pyrvinium pamoate may be more efficient at inducing petites because it is a dipyrvinium salt that has two DNA-binding sites per pyrvinium molecule, and this may result in more efficient binding to DNA nicking-closing enzymes such as the topoisomerases I and II.

13. The primary mechanism of anthelmintic action of pyrvinium pamoate and ethidium bromide may be the destruction of the mitochondrial DNA of helminths.

14. The elevated levels of micronuclei that were obtained in the bone marrow cells of mice after an intraperitoneal injection of pyrvinium pamoate suggest that pyrvinium cations that enter the bloodstream from the gastrointestinal tract could also induce chromosomal damage.

15. It is believed that pyrvinium pamoate does not cross the barrier between the gastrointestinal tract and the bloodstream (Rollo, 1980), however, it is known that monopyrvinium salts can cross this barrier (Hales and Welch, 1953).

16. Pyrvinium pamoate penetrated into the epithelial lining of the crypts in the colon cells of rats (Figure 11), and it induced nuclear aberrations in the colon cells of mice (Figure 12). The results of these *in vivo* mammalian assays suggest that pyrvinium pamoate should be viewed as an anthelmintic drug with the potential for genetic activity in humans.

17. The mechanism of anthelmintic action of the piperazine salts and pyrantel pamoate is the production of a neuromuscular blockade in the parasite, and this effect is also exerted on the host. The piperazine salts and pyrantel pamoate cause side effects that are characterized by headaches and dizziness. This is especially critical in individuals with renal dysfunction; young children, who are the intended consumers of anthelmintic drugs, have compromised kidney function.

18. Mebendazole and thiabendazole cause severe gastrointestinal side effects, and pyrvinium pamoate is mutagenic.

The consequences of untreated, or inadequately treated, pinworm infestations can include the progression of the infestation into the bladder and, more commonly, secondary bacterial and/or fungal infections. When these potential consequences, as well as the risks and benefits of all of the anthelmintic drugs are considered, highly purified pyrvinium pamoate, although mutagenic, should not be excluded as an agent in oxyuriasis therapy.

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